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IMMUNE THERAPY IN MULTIPLE MYELOMA

Can iNKT cells be targeted?

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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

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Scientific output

Mérédis Favreau, Karin Vanderkerken, Dirk Elewaut*, Koen Venken*, Eline Menu*. *Does an NKT cell-based immunotherapeutic approach have a future in multiple myeloma? Oncotarget* 2016 Apr 26;7(17):23128-40. doi: 10.18632/oncotarget.7440. IF_{2015}: 5,008 , **Q1**

Mérédis Favreau†, Eline Menu†, Djoere Gaublomme†, Karin Vanderkerken, Sylvia Faict, Ken Maes, Elke De Bruyne, Srinath Govindarajan, Michael Drennan, Xavier Leleu, Kristin Jochmans, Lennart Zabeau, Jan Tavernier, Koen Venken*, Dirk Elewaut* *Leptin receptor antagonism of iNKT cell function: a novel strategy to combat multiple myeloma. Leukemia* 2017 May 11. doi: 10.1038/leu.2017.146. IF_{2015}: 12,104, **Q1**

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List of abbreviations

A

ACT	adoptive T cell therapy
α-GalCer	alpha-Galactosylceramide
Ang	angiopoietin
aP2	adipocyte protein 2
APC	antigen presenting cell
APRIL	A proliferation-inducing ligand
ASCT	autologous stem cell transplantation
AZA	5-azcytidine

B

BAT	brown adipose tissue
BCMA	B cell maturation antigen
β-GalCer	β-Galactosylceramide
β-GlcCer	β-Glucosylceramide
β2m	beta 2 microglobulin
BM	bone marrow
BMSC	bone marrow stromal cell

C

CAF	cancer-associated fibroblast
CAR	chimeric antigen receptor
CARMA1	caspase recruitment domain-containing protein 11
CBl-b	Casitas B cell lymphoma-b
CCR	C-C chemokine receptor
C-FLIPL	c-Fas-associated death domain-like-IL-1-converting enzyme-like inhibitory protein-long
cIAP2	cellular inhibitor of apoptosis 2
CT-11	pidilizimab
CTLA-4	lymphocyte-associated protein 4
CXCR4	C-X-C chemokine receptor type 4

D

DC	dendritic cell
DKK-1	Dickkopf-related protein 1
DNMTi	DNA methyltransferase inhibitor

E

Egr	early growth response proteins

F

FAK	focal adhesion protein
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization

G

Gadd45β	growth arrest and DNA-damage-inducible 45β
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRAIL	gene related to anergy in lymphocytes

H

HDAC	histon deacetylase
HDACi	histon deacetylase inhibitor
HGF	hepatocyte growth factor
HIF-1α	hypoxia inducible factor
HLA	human leukocyte antigen
HMCL	human myeloma cell line
HMTi	histon methylation inhibitor

I

ICAM-1	intercellular adhesion molecule-1
Ig	immunoglobulin
iGB3	isoglobotrihexosylceramide
IGF-1	insulin-like growth factor 1
IL	interleukin
IMiDs	Immunomodulatory drugs
IMWG	International Myeloma Work Group
iNKT	invariant natural killer T
IP10	ligand interferon gamma-induced protein 10
ISS	International Staging System
iFISH	interphase fluorescent in-situ hybridization
IFN-γ	interferon gamma

J

JAK/STAT	janus kinase and signal transducers and activators of transcription

L

LAG-3	lymphocyte-activation gene 3
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
lyso-PC	lysophosphatidylcholine

M

mAb	monoclonal antibodies
MAPK/ERK	mitogen-activated protein kinases/extracellular signal-regulated kinases
MCL-1	myeloid cell leukemia sequence 1 protein
MCP	monocyte chemotactic proteins
MDM2	murine double minute 2
MDSC	myeloid-derived suppressor cell
MEKK1	mitogen and extracellular kinase kinase 1
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1α
MICA	MHC class I polypeptide-related sequence A
MM	multiple myeloma
MMP	matrix metalloproteinase
MMSET	multiple myeloma SET domain
MRD	minimal residual disease
MSC	mesenchymal cells
mSMART	Mayo Stratification for Myeloma And Risk-adapted Therapy
MUC-1	mucin-1 antigen

N

NCAM	neural adhesion molecule
NCR	natural cytotoxicity receptor
NFAT	nuclear factor of activated T cells
NK	natural killer
NKG2D	natural-killer group 2, member D
NKT	natural killer T
NU-α-GalCer	naphthylurea 6"-derived α-GalCer

O

OPG	osteoprotegerin
OS	overall survival

P

PD-1	programmed death 1
PD-L1	programmed death ligand 1
PDMB5	proteasome subunit β5
PIs	proteasome inhibitor
PI3K/Akt	phosphatidyl-inositol 3 kinase
PPP	picropodophyllin
PRR	pattern-recognition receptor

R

RANKL	receptor activator of NF-κB ligand
Runx-2	Runt-related transcription factor 2

S

Satb1	special AT-rich sequence-binding protein-1
SCID	severe combined Immunodeficient
SDF-1α	stromal derived factor-1 alpha
sFRP-2	soluble frizzled related protein 2
SLAMF7	signaling lymphocytic activation molecule F7
sMM	smoldering multiple myeloma

T

TAMs	tumor-associated macrophages
TCR	T cell receptors
TGF-β	transforming growth factor beta
Th	T-helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin and mucin-domain containing-3
TLR	toll-like receptor
TNFα	tumor necrosis factor-alpha
Treg	regulatory T cells
TSC1	tuberous sclerosis 1

V

VEGF	vascular endothelial growth factor
VISTA	V-domain immunoglobulin-containing suppressor of T cell activation
VLA-4	very late antigen 4

W

WAT	white adipose tissue
WIF-1	Wnt inhibitory factor-1
Wnt	Wingless-type

X

XBP-1	X-box binding protein 1

Y

YAT	yellow adipose tissue

General summary

Multiple myeloma (MM) is a plasma cell cancer where, despite the major therapeutic advances, still the majority of patients relapse and develop a non-responsive disease. Consequently, there's an urgent need for the development of new treatments. In the bone marrow microenvironment of MM patients most of the immune cells are numerically and functionally impaired, such as the invariant natural killer T (iNKT) cell. iNKT cells are unconventional lipid antigen reactive T cells bearing an important role in anti-tumor immunity. Mechanisms behind their deficiency still remain enigmatic and controlling iNKT cell activity has been challenging so far, affecting their implications in immunotherapy. Therefore, we aimed to unravel and overcome possible mechanisms causing iNKT cell deficiency in MM.

We were the first to reveal the leptin – leptin receptor (LR) axis as an important iNKT cell-mediating pathway in MM and anti-tumor immunity. Progressive increase of leptin serum and LR expression levels were observed on iNKT cells during MM progression, both in human disease and the 5T33MM model. Functional analyses proved leptin to profoundly inhibit the iNKT cell functionality *in vitro* and *in vivo*. iNKT anergy, a major drawback in iNKT-based therapies, was strongly alleviated by blocking LR signaling and an almost complete tumor protection in the 5T33MM model was observed when iNKT cells were stimulated in presence of a LR antagonist. Finally, by means of intravital microscopy, we provided for the first time a detailed time course of iNKT cell dynamics in response to α-GalCer. In the second part of the PhD project, the implication of checkpoint molecule PD-1, in mediating iNKT cell deficiency and deficiencies in mucosal-associated invariant T (MAIT) cells, another invariant T cell subset, was evaluated. Not only iNKT cells but also MAIT cells proved to be numerically and functionally impaired in MM, a novel observation that hasn't been reported before. Moreover, we discovered MAIT cell deficiency to be correlated with reduced iNKT cell numbers in MM patients. MAIT cells showed poor activation by α-GalCer-stimulated iNKT cells, illustrating a dysfunctional interaction between MAIT and iNKT cells. Remarkably, elevated PD-1 levels were found on both iNKT and MAIT cells. Treatment with a combination of PD-1 blockade and iNKT stimulation was able to significantly rescue the functionality of iNKT and MAIT cells and conferred superior tumor protection in the 5T33MM model.

To conclude, the work presented here uncovers new promising ways to enhance the anti-tumor function of iNKT cells by restoring them from their state of anergy in MM. The results are groundbreaking in the fight against cancer, as they reveal a new form of cancer immunotherapy, potentially shining a light on future therapeutic avenues for aggressive cancers with currently limited treatment options as well.

Algemene samenvatting

Multipel myeloom (MM) is een plasmacel kanker waarbij, ondanks de grote therapeutische vooruitgang, de meeste patënten nog steeds hervallen door ontwikkeling van resistentie aan huidige therapieën. De nood aan nieuwe geneesmiddelen voor de behandeling van MM is bijgevolg groot. Binnen de MM beenmerg micro-omgeving zijn de meeste immuuncellen functioneel deficiënt, waaronder ook de invariante natural killer T (iNKT) cel. iNKT cellen zijn niet conventionele lipid antigeen reactieve T cellen met een belangerijke rol in anti-tumor immuniteit. In MM zijn ze echter sterk gereduceerd in aantal, alsook functioneel defect. De ongekende mechanismen achter hun deficiëntie en het controleren van de iNKT cel activiteit in MM blijft tot op heden een grote uitdaging en een obstakel in hun gebruik als immmunotherapie. Bijgevolg hadden we tot doel om de mogelijke mechanismen die leiden tot iNKT cel deficiëntie in MM te ontrafelen en te overwinnen.

Als eerste onthulde we de leptine - leptine receptor (LR) as als een belangrijke iNKT cel-gemedieerde pathway in MM en in anti-tumor immuniteit. We toonden, zowel in het 5T33MM muismodel als in MM patiënten, een progressieve stijging van zowel serum leptine als LR expressie levels aan op iNKT cellen. Functionele analyses bewezen dat leptine de iNKT cel functionaliteit zowel *in vitro* als *in vivo* sterk inhibeerde. iNKT anergie, een grote problematiek in iNKT gebaseerde therapieën, werd ook sterk opgeheven door blokkade van de LR signalisatie. Het gebruik van een LR antagonist in combinatie met iNKT stimulatie leidde tot een spectaculaire tumorprotectie in het 5T33MM model. Tot slot, konden we door middel van intravitale microscopie, voor het eerst een gedetaileerd tijdsverloop van de iNKT-celdynamiek als reactie op α-GalCer visualiseren. In het tweede deel van dit doctoraatsproject werd de implicatie van checkpoint molecule PD-1, in iNKT cel deficiëntie alsook bij de mucosaal-geassocieerde invariante T (MAIT) cel, een andere invariante T cel, in MM bekeken. Naast iNKT cellen bleken ook MAIT cellen in MM deficiënt te zijn in aantal en functie. Bovendien was de MAIT cel deficiëntie gecorreleerd met de gereduceerde iNKT cel aantallen in MM patiënten. MAIT cellen vertoonden een verminderde activatie door α-GalCer-gestimuleerde iNKT cellen, wat wees op een dysfunctionele interactie tussen MAIT en iNKT cellen. Opmerkelijk, waren de PD-1 levels op zowel de MAIT als iNKT cellen verhoogd. Combinatie van PD-1 inhibitie en iNKT stimulatie leidde tot een significant herstel van de iNKT en MAIT cel functionaliteit en resulteerde in een superieure kankerbescherming in het 5T33MM model.

In conclusie, tonen we in dit werk nieuwe veelbelovende manieren aan om de anti-tumorale iNKT functionaliteit in MM te verbeteren door hun anergische toestand te herstellen. De resultaten zijn baanbrekend in de strijd tegen kanker, aangezien ze een nieuwe vorm van kankerimmunotherapie onthullen, die ook een hoopgevende therapeutische optie kan vormen voor andere aggressieve kankers met beperkte behandelingsmogelijkheden.

Chapter I

General introduction on multiple myeloma

1. Multiple myeloma

Multiple Myeloma (MM), also known as Kahler's disease, is an incurable monoclonal B cell malignancy that develops in the bone marrow (BM)[1]. MM accounts for approximately 1% of all cancers and is the second most prevalent hematologic malignancy after non-Hodgkin lymphoma[2,3]. This uncontrolled accumulation of terminally differentiated plasma cells in the BM is characterized by an overproduction of monoclonal antibodies (mAb) in the serum and/or urine, also known as M-proteins or paraproteins. Immunoglobulins (Ig) G and Ig A are the two most common types of paraproteins detected in MM patients. Moreover, other major clinical manifestations such as bone lesions, kidney failure, anemia, hypercalcemia and a high risk of infections further typically characterize this disease[1,4].

More common in men than in women, MM is twice more present in the African-American populations compared to Caucasians. However, the reason of this uneven race and sex distribution is still unknown[5]. In Europe, the MM incidence is 4,5 to 6,0 per 100.000 each year with a mortality rate at 4,1 per 100.000. In Belgium around 600 people are diagnosed each year. The median age at diagnosis is situated between 60-65 years[6]. Also obese people (BMI >30), people with the Acquired immune-Deficiency Syndrome (AIDS) and persons with a familial history of lympho-hematopoietic cancers appear to be more prone to develop MM[7–9].

Almost all of the MM patients evolve from an asymptomatic pre-malignant stage called monoclonal gammopathy of undetermined significance (MGUS), which is present in around 3% of the population above 50. The progress to the MM stage happens at a rate of 1% per year. MGUS is an asymptomatic condition where less than 10% clonal plasma cells are found in the BM, where paraproteins can be detected in the blood and/ or the urine (<3g/dL) but in absence of any end-organ damage and further clinical signs of MM[5]. In certain patients, even a more advanced intermediate stage, named smoldering multiple myeloma (sMM) can be observed[10]. The risk of progression from sMM to symptomatic MM is 10% per year and is influenced by the underlying cytogenetic type of the disease. Patients with t(4;14) translocation, 17p deletion, and 1q amplification appear to have a higher risk of progression from sMM to MM[11].

2. The pathogenesis of multiple myeloma

2.1 The normal plasma cell in the bone marrow niche

MM cells represent the malignant equivalent of terminally differentiated, long-lived plasma cells[12]. The development of normal B cells from pluripotent hematopoietic stem cells which differentiate into pro-B cells occurs in the BM. Subsequently, rearrangements of the light- and heavy chain Ig genes occur, which results in the maturation of pro-B cells into naive B lymphocytes. Afterwards, the cell leaves the BM to migrate as mature B cell towards the secondary lymphoid tissues. Due to antigenic stimulation in the germinal center of the lymph nodes a differentiation of the mature B cells into short-lived plasma cells or centroblasts takes place. They then develop into centrocytes and undergo somatic hypermutations and isotype switching resulting in the generation of B cells or plasmablasts that secrete high affinity antibodies. Thereafter, plasmablasts migrate back to the BM where they differentiate into non-dividing, long-lived plasma cells[12,13].

A specific BM niche is required for the long-term survival of normal plasma cells. Plasma cells need stromal feeder cells and an assortment of cytokines to survive. CXCR4 and its ligand stromal derived factor-1 alpha (SDF-1α) expressed on BM stromal cells (BMSCs) are important regarding the homing of plasma cells towards the BM, the plasma cell survival and Ig production[14]. Moreover, BMSC also express IL-6 that through upregulation of anti-apoptotic proteins such as myeloid cell leukemia sequence 1 protein (MCL-1) seems to be crucial for the long term survival of the plasma cells[15]. The transformation of a normal plasma cell to a malignant MM cell involves a process of subsequent oncogenic events occurring due to genetic alterations[12].

2.2 The homing of myeloma cells in the bone marrow niche

One of the key features of MM is the “homing” to the BM. Different studies indicated that MM cells home to the BM through a process called endothelial migration, generally used for the transendothelial migration of normal leucocytes. In this process the first step includes the adhesion of MM cells to endothelial cells which is possible through the expression of multiple adhesion molecules. MM cells are known to express integrins which help in the adhesion to endothelial cells that leads to an arrest of the MM cells. These integrins include the very late antigen 4 (VLA-4) and the lymphocyte function-associated antigen-1 (LFA-1)[16]. In addition, our group demonstrated the involvement of CD44v10 in the endothelial cell adhesion. After integrin-mediated arrest, chemokine receptors present on MM cells and their ligands in the BM microenvironment promote the migration and selective homing of the MM cells[17]. The different chemokine receptors and ligands that were identified to participate in this process include CXCR4 with the ligand SDF-1α, C-C chemokine

receptor (CCR)-1 and CCR5 with ligand macrophage inflammatory protein 1α (MIP-1α), CCR2 with ligand monocyte chemotactic proteins (MCP-1, 2, 3) and CXCR3 with its ligand interferon gamma-induced protein 10 (IP10)[18–21].

Subsequently, there is the transmigration through the BM endothelium and the subendothelial basement membrane. MM cells possess a strong invasive capacity. Through secretion of matrix metalloproteinase (MMP)-2, -9 and urokinase-type plasmininogen activator (uPA), MM cells are able to degrade the extracellular matrix and the basement membrane, consequently helping them to invade and transmigrate into the BM[22]. Moreover, the bone marrow constitutes a hypoxic environment and hypoxia has been shown to benefit MM cell invasion in the BM[23].

2.3 (Epi)genetics and cellular heterogeneity in multiple myeloma

2.3.1 Genetic alterations

MM is known to be a heterogeneous disease characterized by intra-tumor heterogeneity on the genetic and cellular level. Chromosomal aberrations, translocations and mutations are commonly seen in MM. The genetic alterations can contribute to plasma cell immortalization (primary events) or disease progression (secondary events). Several primary genetic defects, being hyperdiploid or non-hyperdiploid events, are observed in MGUS and MM and are considered to initiate MM development[24,25]. Hyperdiploid MM cells are defined by multiple trisomies of odd chromosomes (3, 5, 7, 9, 11, 15, 19 and 21) and are present in 50% of the MM patients[26,27]. The other half has non-hyperdiploid MM cells of which the majority has chromosomal translocations. This constitutes rearrangements of Ig heavy chain promotors to an oncogene, subsequently resulting in the overexpression of that oncogene. The chromosomal translocations can be categorized in three groups. The cyclin D group represents the first group and consists of 18% of the MM patients and targets cyclin D1 [t(11;14)], cyclin D2 [t(12;14)] or cyclin D3 [t(6;14)][24,28]. Secondly, the musculoaponeurotic fibrosarcoma (MAF) group, present in approximately 8% of the MM cases, the translocations result in the overexpression of MAF [t(14;16)], MAFA [t(8;14)] or MAFB [t(14;20)]. The third group covers about 15% of the MM patients and implies the overexpression of the MM SET domain (MMSET) and the fibroblast growth factor receptor 3 (FGFR3) as a result of t(4;14). The chromosome 13 deletion (del13) can be an additional oncogenic event. Also, secondary translocations and mutations have been involved in MM progression and include deletion of chromosome 17p, gain of chromosome 1q and loss of chromosome 1p or chromosome 13/13q, MYC-upregulations, inactivation or mutations of p53-PTEN and NRAS, BRAF and KRAS mutations but also inactivation of cyclin-dependent kinase inhibitors CDKN2A and CDKN2C[25,29–35]. Different components

of the NF-κB pathway were found to be mutated and possibly contribute to MM progression and drug resistance[36,37].

2.3.2 Epigenetic alterations

It is now widely accepted that besides genetic aberrations, epigenetic abnormalities are important for the initiation and progression of cancer, including MM. Epigenetic modifications constitute changes in the pattern of gene expression that are mediated by mechanisms other than alterations in the primary nucleotide sequence. Most of the epigenetic mechanisms described in cancers such as MM, have been found to include DNA methylation, histone modifications and miRNA[38].

- ***DNA methylation***

DNA methylation can be defined as the covalent addition of a methyl-molecule on the 5' cytosine residue preceding a guanine residue in so called CpG dinucleotides. This reaction is catalyzed by DNA methyltransferases (DNMT) and uses S-adenosyl-L-methionine as a methyl donor[39]. In cancer, the global DNA hypomethylation of repetitive sequences, such as Alu and LINE-1 repeats, gene bodies and intergenic regions has been observed and were shown to be responsible for genomic instability, transposon activation, proto-oncogene activation and loss of normal imprinting patterns. Additionally, site specific CpG hypermethylation of gene promotors were found to result in gene silencing. This has been shown for genes involving cell cycle regulation, cell invasion, growth factor signaling, DNA repair, immune modulation and the regulation of apoptosis[38]. In line with this, global hypomethylation of the LINE-1 and Alu repetitive elements was observed in MM patients compared to healthy subjects[40]. Furthermore, global methylation levels of repetitive sequences decreased upon disease progression and were linked with poor prognosis[41,42]. Moreover, hypomethylation of LINE-1 was found to be associated with genomic alterations such as translocations in chromosome 14 and deletions of chromosome 13q, suggesting that hypomethylation of repetitive elements increases the vulnerability to genomic instability[41]. In another study, methylation patterns were associated with specific cytogenetic subgroups, such as translocations (e.g. t(4;14)) and a hyperdiploid state[42]. Interestingly, genome wide DNA methylation profiling revealed that in MM, hypomethylation of genes occurs in early MM disease and further increase upon disease progression, while hypermethylation of genes seemed to be a limited event[43]. Even though considered as an infrequent event, hypermethylation of genes seems to be associated with the progression of MGUS to MM[42]. Namely, multiple studies have revealed, during the analysis of cell lines and primary MM cells, loci-specific hypermethylations of genes involved in the regulation of apoptosis, cell cycle, DNA repair, osteogenesis, Wnt signaling, signal transduction, tumor suppression, hormone and growth factor signaling, cell adhesion, coagulation, hypoxia signaling, transcriptional repression, regulation of

translation and transcription factors as extensively reviewed in [44,45]. Methylation of genes including SPARC, BNIP3, DAPK, RARβ, EGLN3, DLC-1, CDH-1, DCC, TGF-βR2, CD9 and *p*16 were also found to be related to a poor prognosis in MM[46–53]. The mechanisms of aberrant DNA methylation in MM are still unclear but are probably associated with altered expression levels or activity of DNA methyltransferase (DNMT) enzymes, as well as mutations in accessory proteins and promotor sequences[44].

- ***Histone modifications***

Histone modifications are the result of a complex interplay between different molecules referred to as chromatin modifying proteins that are subdivided in "writers" , "readers" and "erasers"[54]. Writers are enzymes catalyzing the actual modification (histone acetyltransferases, HATs) while readers are proteins containing certain domains that recognize the different types of modifications. Erasers consist of enzymes that are removing histone modifications and belong to the group of histone deacetylases (HDACs). Thus, histone modifications recruit, in a context dependent manner, specific protein complexes consisting of different writers, erasers and chromatin accessory proteins. Thereby, they orchestrate various functions related to chromatin including transcription, DNA repair, chromatin structure and DNA replication[54,55]. The most studied modifications that play a key role in chromatin biology constitute acetylation, methylation and phosphorylation. Diverse mutations and translocations involving "writers" , "readers" and "erasers" of histone marks have been demonstrated to induce alterations in the pattern of histone modifications in cancer[56]. To our knowledge, little is known about aberrations of histone modifications in MM. So far, the best documented example in MM is the translocation t(4;14) which leads to overexpression of MMSET (NSD-2), a histone methyltransferase, in approximately 15% of the MM patients[57]. MM cells with t(4;14) showed higher levels of H3K36me2 (di-methylation of histon H3K4) and lower levels of H3K27me3 (tri-methylation of histon H3K4) compared to non-t(4;14) MM cells[58]. Gene expression profiling revealed that MMSET regulates genes involved in the p53 pathway, apoptosis, cell cycle regulation, DNA repair and adhesion and knockdown of MMSET could negatively affect survival and adhesion of MM cells[58,59]. Genes upregulated by MMSET overexpression displayed the H3K36me2 histon mark without the repressive H3K27me3 histon mark. In contrast, silenced genes were enriched for the repressive histon mark H3K27me3 and depleted of H3K36me2[58]. In relation to gene silencing, we discovered a set of under-expressed genes enriched for the histon mark H3K27me3 in MM patient samples compared to normal subjects[60]. Pharmacological intervention with histone methylation inhibitors (HMTi) or the histone deacetylase inhibitors (HDACi) led to re-expression of these silenced genes and impaired the survival of MM cells both *in vitro* and *in vivo*[60]. Related to the H3K27 histon mark, another member Bmi-1, was shown to be upregulated in MM cells compared to

normal plasma cells. On the functional level, Bmi-1 negatively regulates the expression of the pro-apoptotic gene Bim and thus functions as an oncogene. Knockdown of Bmi-1 decreased survival of MM cells by upregulation of Bim, what demonstrates its potential as a target for epigenetic therapy[61]. Lastly, mutations in WHSC1L1 (NSD-3), MLL1-3 and in the histone demethylase UTX (removes histon mark H3K27me) have been described in MM patients. The importance of these mutations in MM still needs to be resolved[62,63]. To our knowledge, no reports on aberrant HDAC functions in MM have been published yet. Nevertheless, HDACi in MM has been extensively studied as a therapeutic approach, as described below in section 4.3.

- ***microRNA expression***

microRNAs are non-coding RNAs that can silence specific target genes through degradation of mRNA or inhibition of translational repression[64]. Few studies demonstrated a dysregulation of miRNA expression in MM cells and this was shown to be associated with genetic abnormalities, such as IgH translocations, deletions and hyperdiploidy in human primary MM cells[65]. Deregulated miRNAs including miR-15, miR-16, miR-21, miR-106b, miR-19a and 19b, as well as miR-181a and b were detected in MM patients and modulated critical genes associated with MM pathogenesis, suggesting the involvement of abnormal miRNAs in MM[66].

2.3.3 Cellular heterogeneity

Aside from (epi)genetic variations, there is the cellular and phenotypical heterogeneity that is adding an extra level of complexity to MM. Different subpopulations exist in MM which differ in clonogenic potential, phenotype, maturation state, gene expression and response to therapy[67–69]. Normal mature plasma cells express high levels of CD38, CD138 and to a lesser extent CD19. MM cells lack CD19 expression and their expression of CD38 and CD138 varies. Variable expression of CD45, CD20, CD27 and CD117 is seen in MM, while CD56, CD28 and CD33 expression levels are generally increased[70]. The characterization of the different subpopulations is still an active study domain for many researchers. Several clonogenic subpopulations have been observed and seem to include cells with both a B and plasma cell phenotype. The relationship between CD138 expression and the MM propagating capacity is the most studied. Research performed by Matsui et al. demonstrated the presence of $CD138^-$B cells in patient's samples and MM cell lines. A higher clonogenic potential and Ki67 expression could be observed for these cells compared to the typical $CD138^+$ plasma cells. Also the cancer stem cell marker aldehyde dehydrogenase was higher expressed on the $CD138^-$plasma cells[67]. Studies performed by our group in the 5TMM mouse model (for information about the model see section 6 of the general introduction) demonstrated that the tumor-initiating potential is not

only CD138 dependent. $CD138^-$cells were also less sensitive to treatment both *in vitro* and *in vivo*[69]. Therefore, it is of major interest to target both $CD138^-$and $CD138^+$ plasma cells in MM pathogenesis.

2.4 The bone marrow microenvironment in multiple myeloma

The BM is the perfect microenvironment where various cross-talks occur between MM cells and the different BM compartments. Close interactions with the cellular components of the BM, including endothelial cells, osteoblasts, osteoclasts, adipocytes and immune cells, confer survival and growth to MM cells and induce angiogenesis, bone destruction, drug resistance and immune escape, ultimately leading to relapse (Figure 1)[71].

2.4.1 Cytokines and adhesion molecules in multiple myeloma

MM cells and the BM microenvironment are linked by complex interactions mediated by soluble factors and adhesion molecules through a network of autocrine, paracrine and adhesion-mediated signals. They promote MM cell survival and proliferation through four major pathways, being the mitogen-activated protein kinases/ extracellular signal-regulated kinases (MAPK/ERK), janus kinase and signal transducers and activators of transcription (JAK/STAT), the phosphatidyl-inositol 3 kinase (PI3K/Akt) and the NF-κB pathway. A summary of the main cytokines and adhesion molecules is described below[71,72].

- ***Cytokines: IL-6 and IGF-1***

IL-6 is an important factor for the growth and survival of MM cells and high serum levels are correlated with poor prognosis. IL-6 is secreted by cells of the BM microenvironment including fibroblasts, osteoblasts, monocytes, BMSCs and even MM cells themselves[73]. Other growth factors such as IL-1β, vascular endothelial growth factor (VEGF), TNFα, transforming growth factor beta (TGF-β), fibroblast growth factor (FGF) as well as adhesion of MM cells to fibroblasts and the upregulation of CD40 can stimulate the expression and secretion of IL-6. Several signaling pathways are activated when IL-6 binds to the receptor on MM cells and consequently stimulate different mechanisms contributing to MM pathology. The JAK/STAT3 pathway activated by IL-6 upregulates the anti-apoptotic proteins Bcl-X_L and Mcl-1 which results in the stimulation of MM survival, while the MAPK/ERK pathway triggers the proliferation of MM cells[73–75]. The IL-6 activated MAPK pathway stimulates both survival and proliferation by inhibiting cell cycle inhibitors p21 and p27[73,76].

Another crucial factor in MM is the insulin-like growth factor 1 (IGF-1) which is locally produced by fibroblasts and osteoblasts[73,77]. Aberrant expression of its receptor can be found on MM cells in association with poor prognosis[78]. When IGF-1 binds to its receptor, survival and proliferation signals

are promoted through activation of the NF-κB and the MAPK/ERK pathway[78]. IGF-1 can cause phosphorylation and leads to the inhibition of pro-apoptotic proteins (i.e. Bcl-2-associated death promoter) as well as downregulation of BIM expression[79,80]. Cell cycle progression can be promoted by degradation of the cell cycle inhibitor p27[81]. Also the sensitivity of MM cells regarding anti-MM drugs can be decreased by IGF-1[77,82,83]. Our group was able to demonstrate the therapeutic relevance of targeting the IGF-1/ IGF-1 receptor axis, by using an IGF receptor-1 inhibitor picropodophyllin (PPP). Tumor growth as well as angiogenesis was inhibited by PPP and a prolonged survival was observed in the 5T33MM and the 5T2MM murine model (see section 6 for detailed info about the models) and synergistic anti-MM effects were seen combining PPP with a Bcl-2 mimetic (ABT-737) or a histone deacetylase inhibitor (LBH589)[84–87].

- ***Adhesion molecules***

Aside from the different growth factors described above, a lot of adhesion molecules such as syndecan (CD138), CD44, CD51, CD54, CD56, VLA-4, -5 and 6, LFA-1, neural adhesion molecule (NCAM) mediate cell-cell contact with BMSCs. This results in the direct and indirect activation of the signaling cascades already mentioned above. MM cell adhesion to fibronectin also contributes to cell adhesion-mediated drug resistance (see section 5.2), mediated by integrins, and leads to an increased DNA repair and stimulation of anti-apoptotic pathways[88,89].

2.4.2 Bone marrow stromal cells in multiple myeloma

Bone marrow stromal cells (BMSCs) are not fully differentiated stem cells and mainly consist of fibroblast, adipocytes and myeloid-derived suppressor cells (MDSCs). They closely interact with MM cells promoting MM disease progression and resistance. BMSCs communicate with MM cells and the extracellular matrix in the BM, through secretion of cytokines, extracellular vesicles and cell-cell contact, such as type I collagen and fibronectin via syndecan 1 and VLA-4 on MM cells and VCAM-1 via VLA-4 on MM cells or through intercellular adhesion molecule-1 (ICAM-1) via mucin-1 antigen (MUC-1) and LFA-1 on MM cells, inducing cell adhesion-mediated drug resistance[90]. This cross-talk activates different pathways resulting in the upregulation of cell cycle regulating proteins and anti-apoptotic proteins. MM–BMSCs interactions principally trigger the NF-κB pathway and IL-6 secretion by BMSCs which in turn enhances the paracrine production and secretion of IL-6 and VEGF by MM cells[73,91]. The Notch pathway is switched on too, which also induce IL-6, IGF-1 and VEGF secretion and stimulates the MM cell proliferation and survival[73,92]. Moreover, BMSC from MM patients were shown to express several pro-angiogenic molecules, such as VEGF, basic-fibroblast growth factor (bFGF), angiopoietin 1 (Ang-1), transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) and interleukin-1 (IL-1)[73,93].

Similar to solid tumors, BMSCs are known to be able to change into cancer-associated fibroblasts (CAFs) in the presence of MM cells. CAFs comprise a heterogeneous population displaying phenotypes similar to those of myofibroblasts (derived from quiescent fibroblasts that have undergone activation during tissue remodeling in wound healing, fibrosis). They actively participate in tumor growth and metastasis by production of cytokines and chemokines, and the release of pro-inflammatory and pro-angiogenic factors, creating a more supportive microenvironment. CAFs express α-smooth muscle actin, fibroblast-specific protein-1, fibroblast activation protein and platelet-derived growth factor receptor-β[94]. Frassanito et al. were the first to demonstrate increased CAF numbers in MM patients compared to patients in remission or MGUS patients[95].

Recent research demonstrated that interaction between BMSCs and MM cells not only occur through cytokines or MM - BMSC cell contact but also through extracellular micro-vesicles or exosomes. They form a class of membranous vesicles secreted by different cell types in the extracellular microenvironment and mediate long-and short-distance communication through stimulation of target cells. They were recently identified as a novel mechanism for intercellular transfer of genetic information in the form of miRNA in clonal plasma cell disorders such as MM[96,97]. Additionally our lab described that BMSC-derived exosomes mediate bortezomib drug resistance and induce MM survival[97].

2.4.3 Endothelial cells and angiogenesis in multiple myeloma

Angiogenesis is a frequent cause of tumor growth, tumor invasion and metastasis in cancer and also in hematological malignancies such as MM[98,99]. It consists of a multi-step process where new vessels are formed from pre-existing vasculature, i.e. capillaries. This process is possible due to interactions between endothelial cells, the extracellular matrix and the secretion of soluble pro-and anti-angiogenic factors by surrounding tissues. Briefly, the different steps in the process include perivascular detachment of existing vessels, endothelial cell proliferation, matrix degradation, endothelial cell migration and formation of new vasculature. Of course, angiogenesis also occurs in healthy individuals but in a controlled way, while for pathological angiogenesis, like in MM, these different cellular events are dysregulated[99].

In MM, angiogenesis was shown to be correlated with disease activity[99]. A switch in favor of the pro-angiogenic path was observed during disease progression in the 5T2 murine model (for preclinical models see section 6) and appeared to be dependent on the downregulation of CD45 and the VEGF production in MM cells[100]. Furthermore, a poor prognosis of the patients seems to be correlated with an increased microvessel density, which is a measure for angiogenesis[101]. Recently, evidence also demonstrated that BM angiogenesis increased progressively from MGUS to sMM to MM[102]. Research

proved that MM cells together with different cell types such as endothelial cells, fibroblasts but also to a minor extent mesenchymal cells (MSC), macrophages and adipocytes are promoting the pro-angiogenic switch. In MM, angiogenesis is the result of an increased secretion of pro-angiogenic factors stimulating pro-angiogenic signaling. Proteases and pro-angiogenic factors are needed for endothelial cell stimulation and the degradation of extracellular matrix, causing the stimulation of this switch and inducing new vessel formation. Pro-angiogenic factors include hepatocyte growth factor (HGF), angiopoietin-1 and 2 (Ang-1; Ang-2), FGF and VEGF. For the proteases mainly matrix-metalloproteinases 2 and 9 (MMP-2; MMP-9) expressed by MM cells are involved in the angiogenic process[103–105]. A major role in angiogenesis was attributed to VEGF, which is produced by MM cells and BMSC, which is essential for endothelial cell proliferation, differentiation and migration[106,107]. Key microenvironmental factors of MM, such as IL-6 and IGF-1 and the endothelial growth factor FGF stimulate VEGF production and thereby establish para-and autocrine loops reinforcing this vicious circle[108–110].

In addition, two recent studies demonstrated MM cell-derived exosomes as new inducers of angiogenesis[111,112]. Exosomes, cell-derived extracellular vesicles, secreted from a human MM cell line directly promoted proliferation and capillary structure formation and induced angiogenesis *in vivo*. These exosomes promoted the expression and secretion of VEGF, further enhancing angiogenesis[112]. Hypoxia-inducible factor (HIF)-1α is often overexpressed by MM cells due to the hypoxic BM environment, leading to increased secretion of pro-angiogenic cytokines[113,114]. Umezu et al. found that MM cells under hypoxic conditions secreted more exosomes with increased levels of miR-135b which induced increased expression of HIF-1α in endothelial cells, leading to enhanced angiogenesis[111]. Moreover, exosomes obtained from the serum of MM patients clearly promoted the proliferation of human vascular endothelial cells as compared to those from healthy donors or MGUS patients[115].

2.4.4 Osteoclasts and osteoblasts in multiple myeloma

Osteolytic bone lesions are one of the most common symptoms and cause of morbidity during MM disease. This typical bone destruction is due to an unbalance of bone formation and bone resorption. It can be attributed to a dysfunction and decrease of osteoblast numbers, which play an important role in bone formation and to an increase in osteoclast number and activity, resulting in bone resorption. Moreover, osteoclasts were shown to stimulate MM survival by secretion of IGF-1, A proliferation-inducing ligand (APRIL) and other growth factors originating from the bone matrix upon resorption[116,117].

The mechanisms behind this bone destruction can be associated with different pathways. One of these pathways constitutes the wingless-type (Wnt) signaling pathway, which is crucial for the differentiation of osteoblasts and consequently bone resorption. Secretion of Dickkopf-related protein 1 (DKK-1), soluble frizzled related protein 2 (sFRP-2) and Wnt inhibitory factor-1 (WIF-1) are responsible for the inhibition of the Wnt signaling pathway, resulting in the myeloma-related inhibition of osteoblast differentiation and activity[118,119]. By means of a paracrine loop mediated by IL-6 and through direct cell-cell interactions between MM cells, osteoblasts and fibroblasts, DKK-1 secretion is induced[120]. Additionally, MM cells are known to express sFRP-2 leading to suppression of bone formation[119,121]. Another mechanism involved in inhibition of osteoblast development is Runt-related transcription factor 2 (Runx-2) activity[122]. Our group could also demonstrate that through deactivation of the Notch signaling pathway the osteoblastic differentiation was impaired in MM-patient derived mesenchymal stem cells[123].

Osteoclast activating factors, including the receptor activator of NF-κB ligand (RANKL), MIP-1α, TNFα, IL-1, -3 and -6 are possible factors responsible in the osteoclast-mediated bone resorption process. RANKL and MIP-1α appear to be the most important ones[116]. Osteoclast formation is induced by RANKL expressing fibroblasts interacting with osteoblasts and RANK expressing osteoclasts, which is prevented by osteoprotegerin (OPG). Thus, osteoclast formation and bone resorption is determined by the balance of RANKL and OPG. This balance is disturbed within MM and consequently correlates with bone resorption. Overexpression of RANKL can be found on the surface of osteoblasts and fibroblasts. OPG, on the contrary, is downregulated when fibroblasts and osteoblasts interact with MM cells[119,124]. Vanderkerken et al. demonstrated that MM progression and survival could be reduced by the use of recombinant OPG[125]. MIP-1α was showed to be secreted by MM cells and was correlated with bone disease and poor prognosis[126]. Recent research has demonstrated MM cell-derived exosomes as mediators of osteoclast formation and activation. MM-derived exosomes supported both survival and migration of osteoclast precursors, and induced their differentiation to osteoclasts, as well as their bone resorption activity. Moreover, exosomes obtained from the plasma of MM patients exhibited the same functions in osteoclast differentiation[127].

2.4.5 Adipocytes

Till now, little attention has been given to the role of the adipocytes in the MM BM cavity. Absent in the BM of a new-born individual, their number increases with aging. This results in adipocytic deposits occupying up to 70% of the BM cavity in the elderly[128,129]. Adipocytes arise from MSCs and are a major type of BMSCs[130–132]. They are the primary component of adipose tissue or “fat”. Adipocytes in white adipose tissue (WAT) contain a single large membrane-enclosed lipid droplet,

surrounded by a thin rim of cytoplasm, and express specific proteins such as adipocyte protein 2 (aP2)[133]. They produce triglycerol and adipokines such as leptin and adiponectin, and serve as a major energy reserve[134–136]. The adipocytes in brown adipose tissue (BAT) contain a central nucleus, many small lipid droplets and mitochondria, and provide a vital source of heat to maintain body temperature (thermoregulation), especially in newborns and hibernating animals. Yellow adipose tissue (YAT) has a mixed characterization and gene expression pattern of WAT and BAT[132]. BM fat is categorized as YAT, with the abilities to initiate osteogenesis and provide energy in emergency situations[137]. A link between adipocytes and cancer was reported in several cancers. Adipocytes are believed to promote rapid cancer cell proliferation in ovarian cancer and induce tumor metastasis and inhibit apoptosis in breast cancer and leukemia[138–140]. Also in prostate carcinoma adipocyte secreted factors were demonstrated to enhance the aggressiveness of the cancer[141]. Clinical studies showed an association between BM adipocytes and an increased risk of MM, but specific biological mechanisms have yet to be elucidated[142,143]. Results demonstrated that adipocytes isolated from femoral BM biopsies supported MM cell proliferation and migration. However, few studies have determined the role of BM adipocytes in MM. Our group could demonstrate the contribution of adipocytes in the BM microenvironment by affecting proliferation, apoptosis and migration in MM. As MM cells invade the BM and have a diffuse growth pattern, BM adipocytes disappear during disease development, suggesting a role of BM adipocytes at the initial stage of the disease, before full remodeling of the BM microenvironment has occurred[128].

Leptin, a product of the obese gene, is a multifunctional cytokine predominantly produced by adipocytes. Different studies reported the effects of leptin on cancer cell growth, migration and invasion, suggesting that this hormone is capable of promoting aggressiveness of the cancer phenotype[141,144]. We showed that adipocytes were the only cells within the MM microenvironment secreting leptin[128]. In MM biology, a possible functional involvement of leptin was not published yet. We revealed that leptin promotes the interactions between BM adipocytes and MM cells. However, it didn't appeared to be the predominant growth factor, suggesting that other growth factors are equally or even more important[128]. Leptin serum levels are reported to be increased in MM patients at diagnosis compared to healthy individuals. Patients with weak leptin receptor expression also tend to have a longer progression-free survival compared to patients with strong leptin expression[145]. Yu et al. recently demonstrated that upregulated leptin could stimulate proliferation of MM and reduce the anti-tumor effect of chemotherapy possibly via the activation of AKT and STAT3 pathways[146].

It becomes more and more clear that adipocytes should no longer be considered as a passive cell in the BM microenvironment and more research could be of great interest.

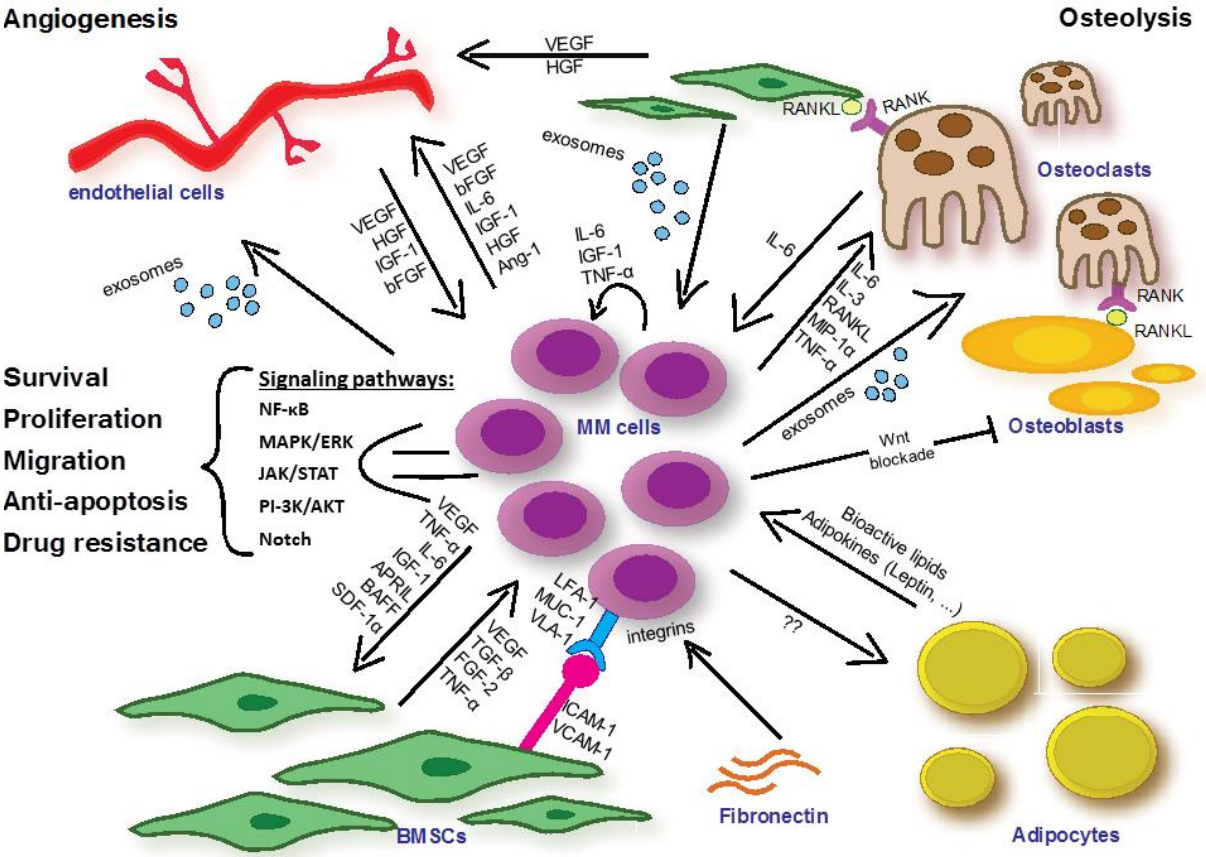

Figure 1: General overview of the interactions between MM cells and the bone marrow microenvironment. *Within the bone marrow MM cells interact through autocrine and paracrine loops with several cell types resulting in angiogenesis, osteolysis and MM disease progression. The interactions are mediated by cell-cell contact, cytokines and growth factors. Adapted from* [71]

2.4.6 The immune microenvironment in multiple myeloma

- ***Myeloid derived suppressor cells***

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous mixture of myeloid cells in different maturation stages that expand in the blood and lymphoid organs of tumor-bearing animals and cancer patients. In mice, MDSC are characterized based on dual expression of CD11b and GR1 and further classified into $Ly6G^{low}$ (monocyte morphology, MO-MDSC) and $Ly6G^{high}$ cells (polymorphonuclear morphology, PMN-MDSC, or granulocyte MDSCs, G-MDSC)[147]. In humans, they are characterized as being $CD33^+$ (a common myeloid marker) and $CD11b^+$ cells without a marker for mature lymphoid or myeloid on their membrane including HLA-DR. They can be subdivided into two main groups based on CD14 positivity. Namely, the granulocyte MDSCs (G-MDSCs) being characterized by $CD11b^+$ $CD14^-$ $CD33^+$ $CD15^+$ HLA-$DR^{-/low}$ and the monocytic-MDSC (M-MDSCs) that can be distinguished by a $CD11b^+$ $CD14^+$ $CD33^+$ HLA-$DR^{-/low}$ profile[147]. The most important feature of MDSCs is regulating the immune system by their immunosuppressive capacity and the promotion of tumor development in MM. MDSCs inhibit innate and adaptive immunity by regulatory T cell activation and secretion of nitric oxid, reactive oxygen species and immunosuppressive cytokines (e.g. IL-10)[147]. STAT 3 has been identified as the main pathway through which MDSCs expansion is mediated in different cancers. Activation of the STAT3 pathway results in the targeting of genes such as cyclin D1, MYC, surviving, Bcl-Xl, and additionally upregulates myeloid-related proteins (e.g. Ariginase 1, S100A8, S100A9), leading to increased survival and proliferation[147].

Recently, our lab identified distinct subsets of MDSC in the BM of the 5TMM models (for information about the models see section 6.1). In the BM, a clear shift toward a $CD11b^+Ly6G^{low}$ population, typically considered to be the phenotype of MO-MDSC, was observed at the end-stage of the disease in the 5TMM models[148]. In addition, in this MO-MDSC population, different MDSC subsets could be discriminated based on their differential Ly6C expression: inflammatory monocytes ($Ly6C^{high}SSC^{low}$), eosinophils ($Ly6C^{intermediate}SSC^{high}$), and immature myeloid cells ($Ly6C^{intermediate}SSC^{low}$) MO-MDSCs were thus more potent in the inhibition of T cell proliferation compared to G-MDSC[148]. The different subsets in the MO-MDSC population contributed to immunosuppression, with the inflammatory monocytes being described as the most potent inhibitors. Moreover, myeloma-derived MDSC showed an upregulation of iNOS, arginase-1, and IL-10, compared to MDSC derived from naive mice. Specific inhibitors of arginase-1 and iNOS partially abrogated the immunosuppressive function of the MDSCs[148]. Regarding the MDSC population in MM patients some controversy exists. Brimnes and colleagues were the first to rapport an increase of strong suppressive $CD14^+$HLA-DR^-/low MO-MDSCs in the peripheral blood of newly diagnosed MM patients compared to healthy subjects[149]. In

contrast, recent studies demonstrated no difference in the MO-MDSC population, but a significant increase in the immunosuppressive G-MDSC, defined as $CD11b^{+}CD14^{-}CD33^{+}CD15^{+}$ or $CD11b^{+}CD14^{-}CD33^{+}CD15^{+}HLA\text{-}DR^{low}$, in BM and peripheral blood of MM patients[150,151]. Different flow cytometry analyses, limited patient numbers and distinct treatment regimens of MM patients could explain the discrepancy seen in these results.

Besides their role in immune suppression, MDSC also play a pivotal role in bone disease. MDSC are macrophage progenitors which are able to differentiate into osteoclasts. MDSC derived from MM-bearing mice had a higher potential to differentiate into mature and functional osteoclasts *in vitro* and *in vivo* compared to MDSC from control mice. Co-inoculation of 5TGM1 MM cells and MDSC resulted in increased tumor burden and bone lesions. In addition, *in vivo* treatment with zoledronic acid, a potent nitrogen-containing bisphophonate, was able to induce a 30% reduction in MDSC numbers and was associated with a decrease in osteoclastogenesis to control levels[152]. Interestingly, it has been shown that mainly the BM MDSC population is able to differentiate into osteoclasts[153].

Little is known about the role and mechanisms of actions of MDSCs in protecting MM cells from drug assault. Our lab extensively performed research on the subject and data demonstrate MDSC-mediated AMPK phosphorylation could increase survival of MM cells. Moreover, AMPK and MDSC targeting strategies showed promising preclinical results that could be applied for future MM treatment[154]. Various factors, including GM-CSF, COX2, TGF-β, IL-4, IL-6, VEGF produced by MM cells and the BM tumor microenvironment also appeared to be involved in the expansion and activation of MDSCs[147]. Furthermore, it is shown that the hypoxic MM microenvironment can modulate the function and differentiation of MDSCs[155]. Finally, MM-derived exosomes are also reported to be involved in the activation and expansion of MDSCs, enhancing their immunosuppressive functions, thus favoring MM progression[156,157].

- ***T regulatory cells***

T regulatory cells (Tregs) constitute an important subgroup of T cells that are involved in the maintenance of self-tolerance, control of auto-immunity and regulation of T cell homeostasis. They also regulate overall immune responses against infectious agents and tumor cells. Tregs develop during normal T cell maturation in the thymus and represent 5% to 10% of the CD4 T cell compartment in the peripheral blood. They express surface antigens such as CD4 and CD25 surface as well as CTLA-4, CD62L, CD69, CD134, CD71, CD54, and CD45RA. Their suppressive activity can be associated with an expression of the forkhead/winged helix family member forkhead box P3 (FoxP3),

which acts as a transcriptional repressor. Tregs can exert suppression on the basis of cell–cell contact and by secretion of immunosuppressive cytokines such as IL-10 and TGF-β[158].

At present, there is significant debate concerning Treg numbers and function in MM. In most cancers, they appear to be increased and functional, while in MM, the situation remains unclear. Prabhala et al. found significantly reduced numbers of FoxP3 cells in MGUS and MM patients while two other groups reported Treg cells to be increased and associated with a strong inhibitory function in MGUS as well as in MM. Finally, there are also studies showing no difference in Treg numbers in patients versus healthy controls[149,159,160]. The divergence in Treg function and numbers described by the different investigators might be due to differences in assay and purification techniques. Reports show increased functional Treg cells in stem cell harvests compared to other lymphocyte populations. The significance of this in relation to tumor response remains unknown, but this suggests that anti-tumor responses might be improved by the selective depletion of Treg cells before transplantation[161]. In a reconstitution of Treg cells after allogeneic stem cell transplantation in MM, it was found that donor-derived Treg cells expanded and accumulated in the BM of patients who underwent transplantation. Treg cells appeared to expand outside the thymus, had a strong inhibitory capacity and exhibited high levels of TGF-β[162]. The therapeutic implications of Treg cells in myeloma are diverse. In allogeneic transplantation, they might play a role in exacerbating graft versus host disease. However, their removal might not necessarily harm the graft-versus-myeloma effect[163]. Moreover, it is possible that depletion of Tregs might allow an anti-tumor effect to occur, especially if this effect can be enhanced with the adoptive transfer of effector T cells[164]. Conversely, if Tregs are deficient and dysfunctional, this might contribute to the impaired immune system in MM. Moreover, they might be responsible for the clinical observations of an increased incidence of autoimmune diseases and the high incidence of infections, which is associated with a higher morbidity and mortality.

- ***Natural Killer cells***

Natural killer (NK) cells are a heterogeneous group of lymphocytes that initiate innate immune responses and have a strong cytotoxic anti-tumor capacity and several immuno-regulatory properties. In humans, NK cells are defined as $CD3^-CD56^+$ lymphocytes but express varying levels of other surface receptors that dictate their cytotoxic and immune-regulatory properties. In MM, NK cells are increased in PB and BM compared to healthy individuals[165–167]. However, this overexpression is not related to their activity status. The NK cell activating receptors, natural killer group 2D (NKG2D) and natural cytotoxicity receptors (NCRs), are reported to be decreased in MM patients and may contribute to immune escape of MM[168,169]. One of the proposed mechanisms consists of an increase

of soluble MHC class I polypeptide-related sequence A (MICA) levels in the circulation of MM patients, which is one of the ligands of NKG2D. This would trigger the downregulation of the latter and result in impaired lymphocyte cytotoxicity[169]. Another suggested mechanism is the expression of programmed cell death 1 (PD-1) on the NK cells of MM patients. Presentation of the ligand PD-L1, expressed on MM cells, downmodulates the NK cell effect versus the MM effect (for more explanation of the immune checkpoint factor PD-1 see section 4.4.4)[170]. NK cell cytotoxicity in humans is induced by the lack of human leukocyte antigen (HLA) expression on the target cell. In contrast to many tumor types, MM cells often do not show loss of HLA class I, suggesting an extra mechanism by which MM may evade targeting by NK cells. NK cell cytotoxicity can also be mediated through signaling via CD16, which mediates the antibody-dependent cellular cytotoxicity (ADCC) of NK cells. However, in MM patients this is often dysfunctional compared to healthy individuals[171].

- ***Myeloma-associated macrophages***

Macrophages constitute a very heterogeneous population originating from monocytes and possess a unique functional plasticity. They are capable of being immunostimulatory or immunosuppressive and either promote or restrain inflammation. Macrophages can be subdivided into distinct types according to their polarization status: M1 (or “classically activated”) and M2 (or “alternatively activated”). The hallmark of M1 macrophages is their immunostimulatory property by iNOS expression, high secretion of IL-12 and a low IL-10 production. On the other hand, M2 macrophages have increased amounts of IL-10 and low levels of IL-12 and TNFα and are consequently orchestrating immunosuppression[172,173].

Tumor-associated macrophages (TAMs), including MM-associated macrophages, resemble the M2-like macrophage population. They are well known for their abundant infiltration in tumor tissues and their supporting role in tumor progression by impacting on angiogenesis, matrix remodeling and immunosuppression[174]. Generally, high levels of TAMs correlate with a poor prognosis in different cancers, also in hematological malignancies like MM[175–177]. In MM patients with active MM, high percentages of $CD68^+$ macrophages were observed but not in non-active disease, MGUS or healthy controls[175,178]. However, the origin of myeloma-associated macrophages still remains unknown: “Are they coming from circulating monocytes or originating from BM residential precursors?” Myeloma-associated macrophages stimulate the MM pathology in different ways. Firstly, they support MM growth and survival through cytokine secretion of IL-1β and TNFα, which in turn stimulates IL-6 production from mesenchymal stem cells (MSCs)[179]. They also secrete IL-10, another factor with an immunosuppressive capacity and contribute to the inflammatory environment through toll-like receptor mediated recognition. Moreover, we demonstrated that the pro-survival effect induced by

TAMs was associated with activation of the STAT3 pathway, less cleavage of caspase-3, and thus less apoptosis[180]. Secondly, TAMs assist in the angiogenesis and vasculogenesis within MM pathology[181]. They secrete the pro-angiogenic factors VEGF, bFGF, IL-8, TNFα, TGF-β and IL-6 resulting in a positive feedback loop amplifying angiogenesis[174,181]. Finally, they support MM in a cell-cell-contact manner and protect MM cells from caspase-dependent apoptosis by IL-6 secretion and selectin-mediated interactions[178].

- ***Natural killer T cells***

For a detailed description of natural killer T cells see Chapter II.

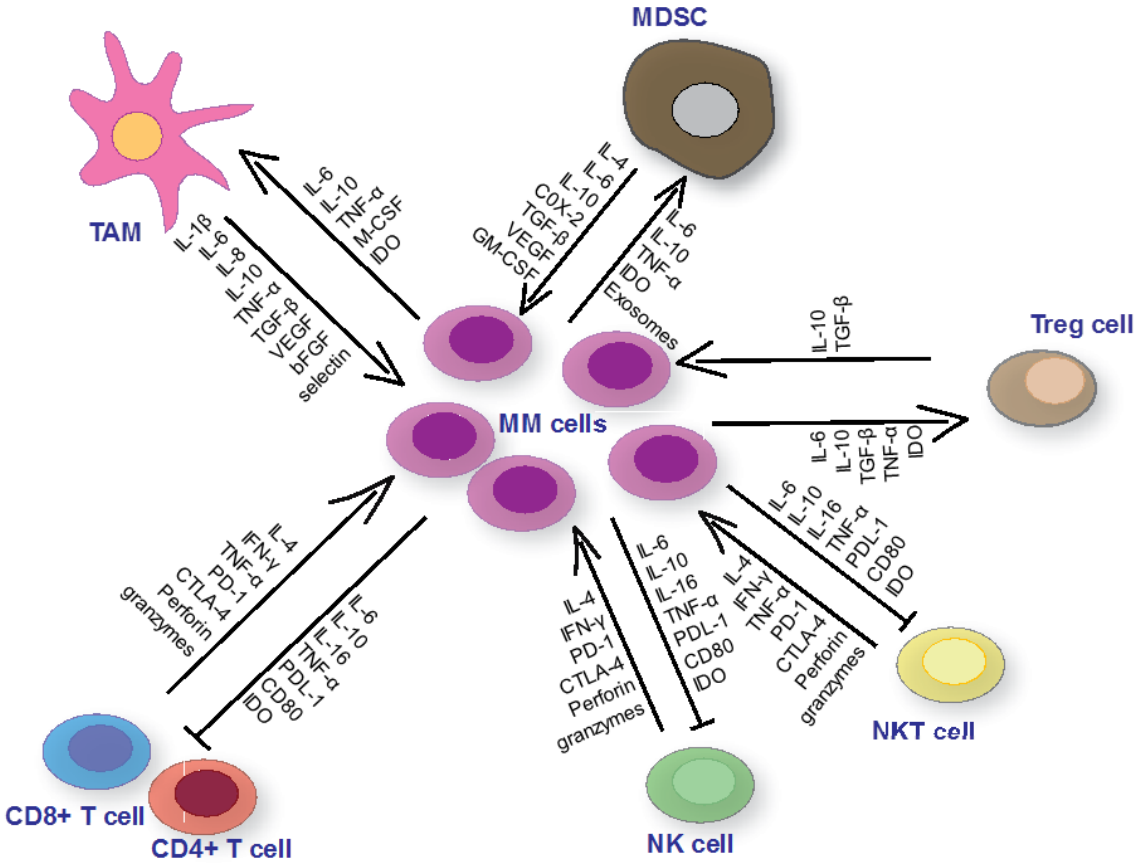

Figure 2: General overview of the interactions between MM cells and the immune microenvironment. Within the bone marrow MM cells interact with several immune cells. Interactions are mediated by cell-cell contact, cytokines and growth factors and are mainly stimulatory for Tregs, MDSCs and TAMs, which are contributing to the proliferation, migration and drug resistance of the MM cells. NK cells, NKT cells and CD8+/ CD4+ T cells fail to eradicate the MM cells. They are suppressed by inhibitory interactions with MM cells, which are also promoting MM pathogenesis.

3. Diagnosis and Risk-Stratification of multiple myeloma

The diagnosis of symptomatic myeloma requires the presence of three main criteria, as shown in Table 1. The first criterium consist of the presence of 10% or more clonal plasma cells infiltration in the BM and/or a biopsy proven plasmacytoma. Moreover, there has to be evidence of MM related end-organ damage, which includes the presence of one or more symptomatic CRAB criteria (hypercalcemia, renal insufficiency, anemia, or bone lesions). Finally, the presence of M-protein in the serum and/ or urine is essential to diagnose MM[182,183].

Table 1: Diagnostic classification of MGUS, sMM and MM

CRITERIA	MGUS	sMM	MM
Serum M-protein	< 3 g/dL	≥ 3 g/dL	Present in serum and/ or urine at any level
Clonal BM plasma cell	< 10 %	≥ 10%	≥ 10% or plasmacytoma
End organ damage	Absent	Absent	**Present CRAP criteria** **C**alcium: ≥ 11,5 mg/dL **R**enal insufficiency: serum creatinine > 2mg/ dL **A**nemia: hemoglobin > 2mg /dL or < 10 g/ dL **B**one lesions: Osteolytic lesions, fractures or sever osteopenia

The prognosis of MM is depending on a range of factors like age, performance status and comorbidities but also on the stage and aggressiveness of the disease as well as the responsiveness to treatment. The International Staging System (ISS), replacing the former Durie-Salmon staging, helps providing prognostic information and is recommended as a standard assessment. The staging system consists of 3 stages (Stage I, II, III) based on beta 2 microglobulin (β2m) and the levels of serum albumin, outlined in detail in Table 2. They give an idea about the inflammatory status and disease activity[184]. Also cytogenic aberrations have an important contribution in determining the prognosis and are useful for both counseling and therapeutic decision-making. Therefore, it's worthy to look with interphase fluorescent *in-situ* hybridization (iFISH) or metaphase cytogenics which type

of aberration is present in the patients. To evaluate the MM risk the International Myeloma Working Group (IMWG) advises to assess the ISS, and to look for t(11;14), t(4;14), t(14;16), t(6;14),t(14;20), trisomies, del17p and gain of 1q21 (Table 3)[185]. Based on the presence of cytogenic aberrations the Mayo Stratification of Myeloma and the Risk-Adapted Therapy (mSMART) Consensus Guidelines defined three risk groups, illustrated in Table 4. Patients with standard risk myeloma have a median overall survival (OS) of 8 to 10 years while those with high risk disease have a median OS of less than 2 to 3 years despite tandem autologous stem cell transplantation (ASCT). For the intermediate risk group the OS is around 4 to 5 years[186]. Many other relevant markers are able to influence the risk for myeloma as well as predict the prognosis and determine the choice of treatment. These factors can be subdivided in 3 groups: tumor biology, tumor burden and patient-related factors. Tumor burden factors include extramedullary disease, while patient-related factors include age, renal function and the Eastern Cooperative Oncology Group performance status (Table 5). For the tumor biology factors it includes ploidy status, 17p-(p53 deletion), t(14;16), t(14,20), t(4,14), deletion 13 on conventional cytogenetic testing, alterations in chromosome 1, t(11;14), t(6;14), lactate dehydrogenase, plasma cell proliferative rate, presentation as plasma cell leukemia and a high-risk signature in gene expression profiling[186].

Table 2: International Staging System (ISS)[184]

ISS	Criteria	Median survival (months)
I	β2m < 3,5mg/l serum albumin > 3g/dl	62
II	β2m < 3,5mg/l or 3,5 -5,5 mg/ml serum albumin < 3g/dl	44
III	β2m > 5,5mg/l	29

Table 3: Risk stratification by the International Myeloma Working Group (IMWG)[185]

Risk	Parameters	Median survival (years)	Patients %
High	ISS II and III, presence of t(4;14), 17p13	2	20
Standard	others	7	60
Low	ISS I and II, absence of t(4;14), 17p13 and no gain 1q21	>10	20

Table 4: Risk stratification of active multiple myeloma[186]

Standard risk	Intermediate risk	High risk
FISH: - t(11;14) - t(6;14)	FISH: - cytogenetic del 13 - hypodiploid - plasma cell labeling index >3%	FISH: - Del 17p - t(14;16) - t(14;20) - high signature gene expression profiling

FISH: fluorescent in-situ hybridization

Table 5: ECOG performance status*

Grade	ECOG
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all selfcare but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited selfcare, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any selfcare. Totally confined to bed or chair
5	Dead

***** *The Eastern Cooperative Oncology Group (ECOG) performance status is a scale used to assess how a patient's disease is progressing, assess how the disease affects the daily living abilities of the patient, and determine appropriate treatment and prognosis.*

The above described criteria used for diagnosis and risk-stratification were partly responsible for the extraordinary evolution in the disease management and treatment of MM patients. Currently, more sensitive techniques are being explored to better capture the genetic diversity of MM as well as improve the clinical management. Applying a "liquid biopsy" blood test as a non-invasive strategy followed by the use of next generation sequencing techniques such as whole-exome and ultra-low pass-whole genome sequencing of cell-free DNA, circulating tumor cells and tumor DNA will enable serial temporal sampling and a better comprehension of the mutational landscape in MM. Moreover, this strategy will offer the possibility to longitudinally follow the dynamics of clonal evolution in MM over time and measure residual tumor burden for new complete remission definitions. However, being in its infancy it is undoubtedly that these strategies represents the future in MM disease monitoring[187,188].

4. Treatment strategies in multiple myeloma

Recent advances in the management of MM significantly improved the treatment outcomes of the patients in the last two decades. The median survival increased from about 2 years in the 1980s up to 5 years in 50 % of the treated patients nowadays (even up to 10 years or longer for another 20%)[189]. The different phases of therapy constitute: initial therapy, ASCT (if eligible), consolidation/ maintenance therapy and treatment of relapse (Figure 3). Unfortunately, MM still remains an incurable disease where the quest for more efficient therapies with durable responses persists.

4.1 Treatment scheme in multiple myeloma

4.1.1 Newly diagnosed patients

- ***Initial therapy: transplant eligible patients***

Depending on the age, the eligibility for ASCT and comorbidities different treatment schemes have been designed. Transplant eligible MM patients (below 65 years) receive an induction therapy of approximately 3-4 cycles of regimens including proteasome inhibitors (bortezomib, carfilzomib), Immunomodulatory drugs (IMiDs; thalidomide, lenalidomide, pomalidomide), glucocorticoids (dexamethasone, prednisone), alkylating agents (melphalan) or anthracyclines (cyclophosphamide, doxorubicin)[190–192]. The most recommended induction therapy consists of bortezomib in combination with dexamethasone together with IMiDs or an alkylator. Bortezomib-based therapies were recently showed to be superior over other treatment regimens in terms of response, progression free survival and overall survival. However, a higher incidence of toxicity was observed[193,194]. After induction therapy, stem cells are harvested, followed by a high-dose therapy of the alkylator melphalan and an ASCT[192].

- ***Autologous stem cell transplantation (ASCT)***

The combination of novel agents together with ASCT showed a strong improvement of the long-term event free survival of patients compared to novel agents alone. At this moment there is no evidence of improved overall survival after early ASCT compared to ASCT at relapse[1,192]. The total therapy approach, including upfront therapy and early ASCT can prevent clonal evolution in MM and resulted in a cure for some patients[195–197]. For high risk patients this strategy is most of the time not enough and novel treatment regimens are necessary. Combination therapies, sequence, single or tandem ASCT, ASCT conditioning and treatment duration are currently under investigation[198–200]. Consolidation and maintenance therapy can be applied in order to enhance the quality of the

response after the transplantation. However, these approaches are not yet approved in Europe[194,201]. A second transplantation can be performed followed by additional cycles of bortezomib in combination with thalidomide and dexamethasone, since this was proven to prolong remission and the occurrence of event free survival. The maintenance approach aims to increase the time of remission after a successful induction therapy. Lenalidomide-based and bortezomib-based maintenance already proved to promote event free survival and overall survival[194,201,202]. However, still major considerations revolve around concerns for toxicity including second malignancies, optimal duration of therapy and selection of agent and cost of therapy.

- ***Initial therapy: transplant non -eligible patients***

Patients over 65 years of age are in general not suitable for transplantation, mainly due to a poor physical condition or other comorbidity factors. For these patients, another strategy is used. Recommended front-line therapies constitute combinatory treatments of melphalan, thalidomide/ bortezomib and prednisone. Other bortezomib-based or IMiD-based combinatory therapies appear to be at least equally effective but have not been approved by the European Medicines Agency yet[1,190]. Maintenance therapies are currently not recommended. However bortezomib-based and lenalidomide-based therapies seem to have beneficial effects[190].

4.1.2 Relapsed refractory patients

Even though many patients acquire a (stringent) complete response, in most of the cases a small population of residual plasma cells, also called minimal residual disease (MRD), remain and are ultimately leading to relapse[203,204]. This small remaining population of malignant plasma cells is undetectable when morphologically assessed in the BM. A lot of efforts have been made the last years in order to improve the detection of MRD such as PCR, multiparameter flow cytometry, positron emission tomography and sequencing[203,204].

Patients are monitored by biochemically identifying M-protein levels in the serum or urine and a follow-up of the CRAB criteria. When the level of M-proteins is significantly increased this can be an indication for relapse. However, treatment is generally started when high M-protein levels are detected even without clinical myeloma-related symptoms, especially in patients who had only a suboptimal response to former treatments and who have a negative cytogenetic profile. The therapy depends on different factors: response to initial therapy after diagnosis, age, presence of neuropathy/ nephropathy, degree of relapse and the cytogenetics[190,205]. In case of a long-term event free survival (>12months) young patients can undergo a second ASCT. If there was a long term remission of more than 1 year and no occurrence of toxicity, it is possible to treat the patient with

the same compounds used in the initial therapy. If this is not the case a switch to other drug classes, IMiD-based or bortezomib-based, are favored. The proposed rationale behind this is that at relapse, the selected clones that grow out are resistant to previous treatment regimens but can possibly still be sensitive to other drug classes. Identifying the clonal architecture at relapse could be useful to re-direct therapy[190,205].

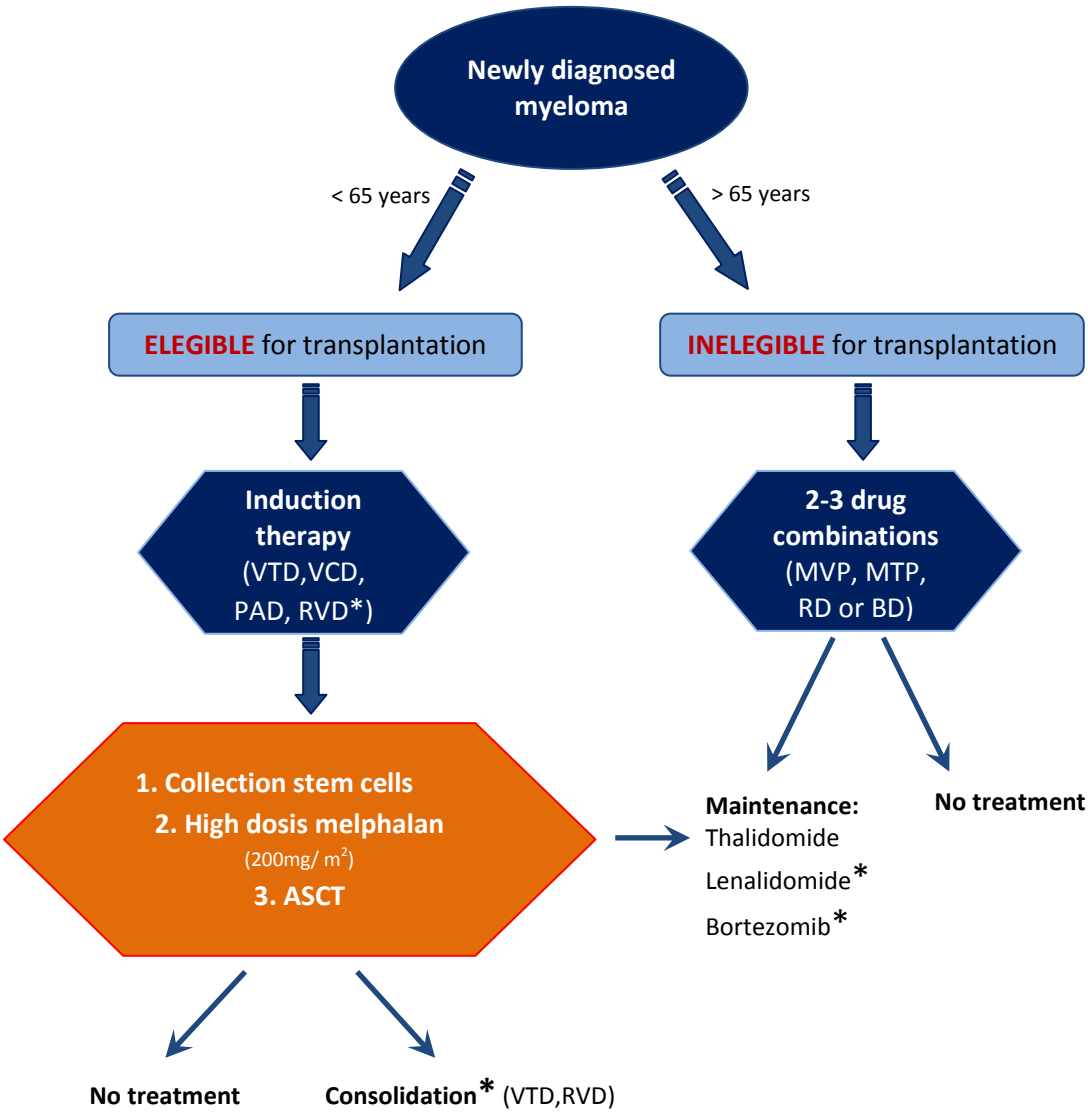

Figure 3: Overview currently used treatments for newly diagnosed myeloma patients. *All drugs present in the treatment scheme are reimbursed in Belgium, except for these indicated with *. VTD: velcade, thalidomide, dexamethasone; VCD: velcade, cyclophosphamide, dexamethasone; PAD: prednisone, adriamycin (doxorubicin), dexamethasone; ASCT: autologous stem cell transplantation; RVD: lenalidomide, velcade, dexamethasone; MVP: melphalan, velcade, prednisone; RD: lenalidomide, dexamethasone; MTP: melphalan, thalidomide, prednisone; BD: bendamustine, dexamethasone. Adapted from* [206,207]

4.1.3 Supportive therapy

In terms of survival, supportive therapy could be beneficial for all MM patients. As previously described, complications such as anemia, bone pain, hypercalcemia/-uricemia and infections, occur in MM[208]. Analgesic agents against pain and antibiotics to fight the multiple infections can be recommended. Both, bone fractures and pain can be reduced by radiotherapy of the osteolytic lesions. Biphosphonates are widely used to treat bone disease. Zoledronic acid and pomidronate for example had a survival benefit and anti-MM effects in symptomatic MM[209,210].

4.2 Proteasome inhibitors

Proteasome inhibitors (PIs) have emerged 10 years ago as an important therapeutic strategy in MM. The ubiquitin-proteasome pathway is responsible for degradation of the majority of regulatory proteins in eukaryotic cells, including proteins that control cell-cycle progression, apoptosis, and DNA repair and therefore plays an essential role in maintaining normal cellular homeostasis. In cancer in general, higher levels of proteasome activity are seen compared to normal cells. Moreover, they are more sensitive to the pro-apoptotic effects of proteasome inhibition than normal cells. MM cells were particularly found to be susceptible to proteasome inhibition because they are proliferative, over-produce defective proteins which need to be degraded by the proteasome and additionally upregulate signaling pathways dependent on the 26S proteasome[211]. Since the first-in-class PI bortezomib, a substantial improvement in survival of MM patients has been observed over the past decade. Recently, second-generation PIs have been developed and are undergoing intense evaluation in clinical trials. Each PI can be distinguished regarding their specificities and affinities for the different catalytic sites within the ubiquitin-proteasome pathway[212]. PIs can be classified into 3 groups, being the boronates, epoxyketones, and salinosporamides, based on their chemical structure and active moiety.

- ***Boronates***

Bortezomib is a dipeptidyl boronic acid-based specific, reversible PI that inhibits the proteasome by mainly targeting the chymotrypsin-and caspase-like active sites[212]. Bortezomib suppresses tumor survival pathways, arrests MM growth, invasion, and angiogenesis[213]. Bortezomib directly induces apoptosis of MM cells, inhibits the activation of NF-κB in cells and in the tumor microenvironment, reduces adherence of myeloma cells to bone marrow stromal cells, blocks production and intracellular signaling of IL-6 in myeloma cells, stops the production and expression of proangiogenic mediators, and overcomes defects in apoptotic regulators, such as Bcl-2 overexpression and

alterations in tumor suppressor p53[213,214]. In addition, bortezomib has shown enhanced endoplasmic reticulum stress, associated with the disruption of an unfolded protein response. As a result of its mechanisms of action, bortezomib has also been associated with increased bone formation and osteoblastic activity, and decreased bone resorption and osteoclastic activity[214–217].

- ***Epoxyketones***

Carfilzomib is an irreversible PI belonging to the category of the epoxyketones. It enhances a potent and sustained inhibition of chymotrypsin-like activity with a greater selectivity for the chymotrypsin-active sites compared to bortezomib. Carfilzomib has been shown to trigger cell cycle arrest, induce apoptosis, and activate stress response pathways in human tumor cell lines, including MM and other hematologic malignancies and solid tumors[218,219]. Importantly, carfilzomib has demonstrated activity against bortezomib-resistant cell lines and primary MM cells[220]. It has a good safety profile with less peripheral neuropathy compared to bortezomib[221]. Carfilzomib has good results in relapsed and refractory MM patients, including those who received bortezomib previously[222]. Moreover, promising combination activity with glucocorticosteroids and IMiDs was shown in NDMM[223].

- ***Salinosporamides***

Marizomib is a natural lactone compound derived from the marine bacterium Salinospora tropica which belongs to the salinosporamides. It is an irreversible PI that, unlike bortezomib and carfilzomib, inhibits both the chymotrypsin-like and trypsin-like protease activities. Marizomib only minimally affects the caspase-like active sites of the proteasome. Marizomib-induced apoptosis is predominantly the result of activation of caspase-8–mediated signaling pathways[224]. Marizomib has demonstrated anti-tumor activity in preclinical models of MM, other hematologic malignancies, as well as in solid tumors[225].

4.3 Epigenetic modulating agents in multiple myeloma

DNA methylation and posttranslational histone modification constitute two epigenetic modifications that have a major impact on MM cell biology and greatly influence development and progression of MM (as previously discussed in section 2.3). Epigenetic modulating agents such as histone deacetylase inhibitors (HDACi) and DNA methyltransferase inhibitors (DNMTi) are therefore under intensive investigation for their therapeutic use in MM.

As demonstrated by us and others, HDACi used alone or in combination with conventional anti-MM agents have a potent preclinical anti-MM activity[226–230]. While single-agent HDACi appeared to mediate little to no clinical activity, combinatory treatment of the pan-HDACi vorinostat or

panobinostat in combination with respectively the proteasome inhibitor bortezomib or bortezomib plus dexamethasone did prolong progression free survival with respectively 0.8 months and 3.9 months[231–235]. However, the clinical relevance in terms of overall survival is not yet clear and a high occurrence of side effects was observed[234,235]. In addition, a phase II trial of the combination of panobinostat with melphalan, thalidomide and prednisone was also associated with a high occurrence of side effects[236].

Aberrant DNA methylation can be targeted with the use of DNMTi leading to demethylation of DNA[45]. Commonly used DNMTi are the cytidine analogues 5-azacytidine (AZA) and 5-aza-2'-deoxycytidine (decitabine). Their anti-tumor effects have been linked to two mechanisms: passive DNA demethylation and induction of DNA damage[237]. The commonly used DNMTi, AZA, has been shown to have anti-myeloma activity by promoting apoptosis and inhibiting IL-6 and NF-κB signaling pathways[45,238]. Additionally, AZA is directly cytotoxic and has been shown to cause double stranded DNA breaks, inducing p53-mediated cell cycle arrest and apoptosis[239,240]. Upon replication, cytidine analogue decitabine is incorporated into DNA thereby trapping DNMT enzymes. This consequently leads to the depletion of DNMT and a loss in ability to methylate DNA. The result is a genome-wide loss of methylation leading to re-activation of silenced genes, genomic instability and related anti-tumor effects. Moreover, activation of a DNA damage response ultimately results in apoptosis[239]. At this moment, DNMTi are considerably less used in the clinic for treatment of MM compared to HDACi. Some clinical trials are ongoing to evaluate the safety of DNMTi and a phase II clinical trial in MM patients combining AZA and lenalidomide before and during ASCT was perfomed[45]. Cytotoxic T lymphocyte reactions targeting the MM cells were observed, demonstrating an adaptive immune response induced by AZA. However, it's clear that further clinical trials are needed to evaluate DNMTi in clinic.

There has also been considerable interest in combining DNMTi and HDACi to enhance the anti-tumor effects of both agents[241]. The mechanism of action involves a broad spectrum of effects that range from true epigenetic changes, chromatin-and DNA-related effects, disruption of the acetylome and micro-environmental effects[45]. Previous studies on these combinations demonstrated alterations in gene expression that may correlate with an enhanced apoptotic effect and direct modulation of downstream apoptotic effectors[46,242–246].

4.4 Immune therapy in multiple myeloma

Fighting cancer by harnessing the body's own immune system has been considered the holy grail in cancer therapy and at present also in MM more and more success is achieved in the field of immune

therapy. It is known that MM cells can evade the immune system through a variety of mechanisms: inhibition of tumor antigen presentation, secretion of immunosuppressive factors, recruitment of immunosuppressive cells and suppression of immune activation. Numerous strategies to modulate this immune evasion, such as passive and active immunotherapy, have been developed and are under evaluation. Passive immunotherapy includes tumor-directed monoclonal antibodies, while active immunotherapy acts directly on the patient's own immune system to trigger an immune attack against cancer. Active strategies can be therapeutic vaccines, cytokines, mediators of T cell activation, etc[247]. An overview of the most relevant immune-targeting strategies in MM is given below.

4.4.1 Immunomodulatory drugs (IMiDs)

IMiDs directly affect the BM microenvironment and MM cells themself. They include thalidomide and its less toxic analogues lenalidomide and pomalidomide. IMiDs have pleiotropic mechanism of actions, resulting in anti-MM effects[248]. They have the capacity to modulate cytokines in the MM microenvironment by inhibiting the release of TNFα, IL-1 and IL-6 and triggering the release of IL-2, IL-12 and IFN-γ cytokines. They also induce inhibition of angiogenesis and increase immune effector cell numbers and functions of cytotoxic T-, NK- and NKT cells possibly via IL-2 and inhibition of immunosuppressive cell populations such as Tregs and MDSCs. By decreasing the expression levels of adhesion molecules on malignant MM cells and BMSCs, IMiDs abrogate interactions between MM cells and their microenvironment and sensitize the cells to other cytotoxic therapies. Moreover, they possess anti-proliferative properties through the upregulation of cyclin-dependent kinase inhibitors (p21, p27) and tumor suppressor genes in primary MM cells including early growth response proteins (Egr-1, -2 and -3). By modulating the NF-κB pathway and the expression of pro-and anti-apoptotic molecules they extra potentiate their anti-cancer properties[249–252].

Cereblon, an E3 ubiquitin ligase component, has recently been identified as a direct molecular target of IMiDs and has been shown to be crucial for the direct teratogenic, cytotoxic and immune related effects of IMiDs. Ikaros family zinc finger proteins 1 and 3 are two downstream transcription factors taking part in the IMiDs-mediated anti-cancer activity[253,254]. All these properties make IMiDs perfect candidates to combine with other anti-MM agents.

4.4.2 Vaccination strategies

- ***Peptide-based vaccines***

Tumor-associated antigens, such as MAGE, NY-ESO1, WT-1, RHAMM-R3 and XBP-1, used as targets were able to induce cellular responses in a preclinical context when they were used individually and/or in combination. Initially, clinical results using single peptide-based vaccines demonstrated that these vaccines could be used with few adverse effects and induced immune responses, however with moderate effects on disease control. A preclinical study using T cells from MM patients researchers were able to generate peptide specific cytotoxic T lymphocytes by using, XBP-1, CD138 and CS1 as immununogens[255]. Currently numerous peptide vaccination trials are ongoing and efforts were made to enhance the immunogenicity of these vaccines in combination with T cell therapy. Impressive immune responses were observed but clinical outcome was lacking[256].

- ***Idiotype-based vaccines***

The monoclonal immunoglobulin or idiotype protein (paraprotein) secreted by myeloma cells carries unique antigenic determinants. Immunotherapy with idiotype-pulsed DCs has been explored as a therapeutic strategy in MM for the past decade but the results have been disappointing. Less than 50% of patients mounted idiotype-specific immune responses, and satisfactory clinical responses have rarely been observed[257–259]. To improve the efficacy of idiotype-pulsed DCs, Yi et al. pulsed them with CD40L. The idiotype-pulsed CD40L-DC vaccines were subsequently injected intranodaly in MM patients. Enhanced Th1 and specific CD8+ T cell responses were induced in 5 out of 9 patients[260].

- ***DC/MM fusion vaccines***

Another vaccination strategy involves DC/MM cell fusion vaccines. The advantage of this approach is the capacity of DCs to present several antigens from cell to host. These vaccines were already evaluated in phase I and II trials. In both trials DC/MM fusion vaccines were well-tolerated and triggered the tumor specific immunity through expansion of reactive CD4 and CD8 T cells and induction of tumor specific antibody responses[261]. In the second study, DC/MM fusion 100 days after ASCT was associated with the depletion of Treg cells. A quarter of the patients developed complete response after vaccination, suggesting that the vaccine induced responses eliminated the minimal residual disease[262].

- ***TriMix-DC vaccines***

In a TriMix-DC-based strategy DCs are electroporated with miRNA's encoding for CD70, CD40L and constitutively active Toll-like receptor 4 in combination with miRNA encoding for TAA to induce co-stimulation with T cells and induce TAA-specific $CD8^+$ cells in patients. Showing successful results in stage III and stage IV melanoma patients, a similar approach was tested in MM by the group of Prof. Thielemans (VUB)[263]. TriMix-DCs proved to be efficient stimulators of T cells from MM patients *in vitro*. Preclinical studies showed promising results of a polarization of activated T cells towards a Th1 response, leading to the start of a clinical trial in the hospital of the UZ Brussels[264].

4.4.3 Antibody therapies

- ***Anti-CS-1(SLAMF7) antibody***

CS-1 or signaling lymphocytic activation molecule F7 (SLAMF7) is a glycoprotein expressed on normal plasma cells, MM cells as well as on NK and NKT cells. This has led to the appealing development of a humanized IgG1 monoclonal anti-CS1 (SLAMF7) antibody called elotuzumab, which has shown remarkable anti-myeloma activity in both preclinical and clinical studies. The monoclonal antibody has a dual mechanism-of-action. It directly activates NK cells via the SLAMF7 pathway and also targets SLAMF7 on MM cells, tagging them for NK cell-mediated destruction via antibody-dependent cellular toxicity[265,266]. Elotuzumab does not show significant clinical activity as a single agent. However, in combination with lenalidomide and dexamethasone high efficacy was demonstrated in relapsed/refractory MM[267–270]. Today, the combination elotuzumab, lenalidomide and dexamethasone is already approved by the U.S. (Food and Drug Administration) FDA for the treatment of patients who have received one to three prior therapies[271].

- ***Anti-CD38 antibody***

CD38 is a type II cell surface transmembrane glycoprotein being present on myeloid and lymphoid cell lineages in the BM, but also expressed on some non-hematopoietic cell types. It has different functions in cell adhesion, signaling and enzymatic activity. Since it is highly expressed on MM cells, CD38 is an interesting target for therapy. Daratumumab is a fully human IgG1κ mAb directed against CD38 that has shown anti-MM activity in different preclinical models, using mechanisms such as antibody-dependent cellular toxicity, complement dependent cytotoxicity and antibody-dependent cellular phagocytosis[265,272]. It also showed extraordinary effectiveness in heavily pretreated MM patients as a single agent with a manageable safety profile[270,273,274]. Based on these phase I/ II trials the U.S. FDA has granted accelerated approval for daratumumab to treat patients who have received

at least three prior treatments making it the first monoclonal antibody approved for the treatment of MM[275]. Currently, daratumumab is investigated in combination with proteasome inhibitors and IMiDs in newly diagnosed and relapsed/refractory MM patients. Since it has a favorable safety profile, clinical studies with daratumumab as maintenance therapy are conducted as well[276]. Several other anti-CD38 antibodies such as SAR650984 (Sanofi), MOR03087 (Morphosys) or Ab79 (Takeda) are currently also in the early phases of clinical development and testing, but daratumumab (Genmab/Janssen) seems to be the most promising[270].

4.4.4 Immune checkpoint inhibitors

One of the most encouraging approaches to activate therapeutic anti-tumor immunity is the blockade of immune checkpoints. Immune checkpoint proteins, such as programmed death 1 (PD-1), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin and mucin-containing protein 3 (TIM-3), are inhibitory molecules that are essential for maintaining self-tolerance and for modulating the duration and amplitude of physiological immune responses in the peripheral tissues in order to prevent collateral tissue damage[277,278]. In a lot of cancers, the expression of immune-checkpoint proteins is dysregulated and used by tumors as an important mechanism to evade the immune system. Because many of the immune checkpoints are initiated by ligand–receptor interactions, they can easily be blocked by antibodies or modulated by recombinant forms of ligands or receptors[279]. Remarkable results have already been observed in melanoma, kidney cancer, lung cancer, colorectal cancer and head and neck cancer[280,281]. Also in hematological malignancies immune checkpoint blockade looks like a promising strategy[282].

The PD-1 protein is a member of the B7 immunoglobulin superfamily and is expressed on T- and B cells, NK cells, DCs and monocytes. Multiple malignancies express the PD ligand 1 (PD-L1) molecule which has been correlated with poor prognosis[281]. MM cells were shown to express PD-L1 while normal plasma cells did not. High levels of PD-1 were observed on NK-and T cells from MM patients and not from healthy individuals[170,283]. These abnormal expression profiles in MM strongly encouraged the further investigation of immune modulation and immunotherapy in MM. Moreover, preclinical investigations showed anti-myeloma activity by inhibiting PD-1/PD-L1 signaling. MM growth was inhibited both *in vitro* and *in vivo* using an anti-PD-L1 antibody[282]. The anti-PD-1 mAb CT-11 (pidilizumab) was tested to evaluate anti-MM effects and was able to enhance NK cell anti-tumor activity. Particularly, in presence of lenalidomide or with DC/MM fusion vaccine, downregulation of PD-L1 on MM cells was demonstrated[170,283]. A phase I study with pidilizumab already demonstrated tolerability in 30% of study patients with hematological malignancies. Currently, a phase II trial is

ongoing to assess efficacy of a DC/tumor vaccine in combination with pidilizumab following ASCT[284]. The phase I clinical trial with mAb nivolumab in MM patients showed no objective responses. Although, results were initially disappointing, disease remained stable for 18 of 27 patients[282]. Multiple other studies using different anti-PD-1 antibodies are ongoing in advanced MM patients and include pembrolizumab with pomalidomide, with lenalidomide/dexamethasone, or post ASCT[285]. Furthermore, a preclinical study noted that combining PD-L1 blockade with other immune checkpoint inhibitors (CTLA-4, LAG-3, or TIM-3) promotes survival of MM mice that underwent a low dose total irradiation[286].

An alternative for immune checkpoint blockade could be the use of agonist mAb, such as anti-CD137, directed against co-stimulatory molecules. Anti-CD137 demonstrated to induce potent T- and NK cell-mediated responses in murine MM models[287]. Currently clinical trials with anti-CD137 mAbs are ongoing to evaluate the safety and beneficial effects in cancer patients[288,289].

Immune checkpoint inhibitors are a promising avenue that should eventually lead to more efficient combination strategies in MM.

4.4.5 Chimeric Antigen Receptor (CAR) T cells

Chimeric antigen receptor (CAR) T cell based therapy has shown great promise in lymphoma and certain kinds of leukemia and are currently also under investigation in MM. CAR T cells are engineered to replace the extracellular antigen binding domain by single chain variable fragments (scFv) of a mAb specific for a surface antigen with an intracellular signaling domain. They are then stimulated to proliferation so that a large number of the altered cells can be re-infused back into the patient and induce an anti-tumor response[290]. Antigen specificity of T cells is dependent on the T cell receptor (TCR) itself and the specificity of the TCR determined by major histocompatibility complex (MHC) molecules. The target killing function of the engineered CAR T cells is MHC-independent. A great advantage since T cell therapy is frequently confronted with tumor escape by MHC mutations[291]. Thus far, the few attempts to use CAR T cell therapy in multiple myeloma have shown limited success. One of the few successful examples is the CD19 targeted CAR T cell approach. Garfall et al. observed a low but more frequent expression of CD19 on malignant plasma cells and targeted this population using CTL019 cells, which are lentiviral transduced autologous T cells harboring the CD3zeta/ CD137 anti-CD19 chimeric receptor. Of the 9 patients involved more than half were in remission[292]. Another example is the B cell maturation antigen (BCMA)-directed CAR T cell. BCMA is uniformly expressed in more than 60% of the MM patients. Since BCMA is only expressed by plasma cells and a small fraction of B cells this looks an encouraging target. 11 patients with advanced MM

and a median of seven previously failed therapies have participated in the phase I clinical trial. Two patients treated at the highest dose had strong anti-cancer responses. One achieved a stringent complete remission, while the other had undetectable MM cells in the BM cells, but had not yet reached the complete remission status. Of the six patients treated on the lowest two doses, one patient experienced a short partial remission and the other five remained stable. Two patients on the second-highest dose maintained stable disease, and one patient obtained a very good partial response[293]. However, there are many challenges and unknowns that have to be taken into account such as cell dosage, toxicity, timing of CAR infusion and what immune cell to use, etc. In MM, many of the potential CAR target antigens have a broad normal tissue distribution. So being cautious is warranted.

5. Drug resistance in multiple myeloma

Drug resistance still constitutes a major obstacle for the successful treatment of MM. Despite the major advances obtained in the field, many patients relapse and become refractory to the available treatments. Resistance can be caused by either intrinsic (Figure 4) or extrinsic BM-mediated mechanisms. Intrinsic resistance is defined as an innate property of the MM cells to resist therapy at diagnosis. This can be caused by mutations, translocations, methylations or microRNA abnormalities[294,295]. However, in general most of the patients initially respond to treatments, but relapse later on and become insensitive to treatment. This is called acquired drug resistance. Below, different mechanisms contributing to MM drug resistance are discussed.

5.1 Intrinsic mechanisms

5.1.1 Drug efflux pumps

The efflux ATP-dependent transporter P-glycoprotein, also referred to as MRD-1 or ABCB1, has been observed to be frequently overexpressed upon exposure to therapeutic agents such as doxorubicin, vincristine, dexamethasone and PIs (bortezomib and carfilzomib), resulting in a reduced accumulation of the drug in the cell and subsequently contributing to resistance[295–299]. In order to overcome this resistance, combinations with P-glycoprotein inhibitors (cyclosporine, verapamil and PS-833) and vincristine, adriamycin and dexamethasone were tested. Unfortunately, no clinical benefits were observed due to poor inhibition of the P-glycoprotein and toxicity[300]. ABCG2, another member of the efflux ATP-dependent transporter family, also appeared to be upregulated upon treatment, however it didn't seem to contribute to multidrug resistance[301]. Recently, Zhou et al. could demonstrate the overexpression of NEK, which is responsible for the upregulation of the ABC

transporter family members (including P-glycoprotein), resulted in drug resistance to bortezomib and doxorubicin, consequently leading to rapid relapse and a poor outcome[302].

5.1.2 Alteration in drug targets and pathways

The upregulation of the IGF-1/ IGF-1 receptor pathway has also been shown to contribute to bortezomib resistance in MM. Addition of IGF-1 reduced the sensitivity to bortezomib. Moreover, knocking down the IGF-1 receptor increased the sensitivity of MM cell lines and primary patients samples to bortezomib. Furthermore, combination of bortezomib and the IGF-1 receptor inhibitor OSI-906 acted synergistically in MM cell lines and murine xenograft MM models, whereas single agent therapy had no effect[82]. β-catenin, key protein in the canonical Wnt pathway, degrades via the ubiquitin-proteasome pathway. MM cell lines with higher β-catenin levels were bortezomib resistant and had higher levels of TCF-4 transcription factor (central player in Wnt signaling). Reduction of cytoplasmatic levels of β-catenin by arsenic trioxide increased the sensitivity of MM cells against bortezomib[303]. The group of Vacca et al. found that the C-MET pathway is constitutively expressed in relapsed resistant MM patients. C-MET phosphorylation in resistant MM cells could be observed[304]. Inhibition of the latter increased apoptosis, downmodulated the proliferation of resistant MM cells and reversed resistance towards bortezomib, melphalan, doxorubicin *in vitro*. *In vivo* tumor growth could be delayed in MM xenograft models[304]. Upregulation of heat shock proteins (HSP) can be the consequence of a down modulation of the ubiquitin-proteasome pathway and has been linked to drug resistance. They contribute to MM survival by maintaining protein homeostasis, blocking apoptosis and stabilizing oncoproteins. Different inhibitors such as HSP-70, HSP-90 and Hsf1, master regulator of heat shock expression, were developed and display promising results in combination with PIs regarding the reduction of MM activity[305–308].

The drug bortezomib induces a full inhibition of ubiquitinated protein hydrolysis through selective binding to the proteasome subunit β5 (PDMB5). *In vitro* studies, demonstrated that resistance to bortezomib has been linked with an overexpression or a point mutation of the PSMB5. Silencing of PSMB5 increased the sensitivity against bortezomib[309]. However, this could not be demonstrated on primary samples or relapsed patients after bortezomib treatment[310]. Activation of P53 has also been considered as an interesting novel treatment target to overcome drug resistance in MM. P53 mutations are present in 2-3% of newly diagnosed patients, with an incidence increasing at later stages of the disease. Patients harboring a p53 mutation are in general resistant to all standard treatments[311,312]. P53 is a crucial tumor suppressor protein responsible for the control of cell cycle, apoptosis, DNA repair, cellular senescence and autophagy. Inactivation of P53 can be the result of mutated or overexpressed murine double minute 2 (MDM2)[240]. MDM2 inhibitors showed anti-

myeloma activity in MM cell lines and primary cells from patients and inhibited MM cell growth in the BM microenvironment[313]. More recently, the oncoprotein mucin 1 C-terminal subunit (MUC-1-C) has been identified as an important drug target to overcome drug resistance. An upregulation of gluthation and TIGAR, the p53-inducible regulator of glycolysis and apoptosis was demonstrated by the authors in bortezomib MM resistant MM cells. Pharmacological inhibition of MUC-1 proved to be successful in the reduction of TIGAR and gluthation and increased oxidative injury and cell death in bortezomib resistant MM cells[314]. Approximately 20% of the MM patients harbor genetic lesions in genes of the NF-κB pathway leading to uncontrolled NF-κB activation and loss of functional TRAF3, being one of the most common deleted/ mutated genes[37]. Our lab recently reported the cellular inhibitor of apoptosis 2 (cIAP2) as being an important player in resistance of MM cells harboring TRAF3 deletions to proteasome inhibition[37]. Resistance was caused by a decrease of cleaved caspases upon treatment, activation of the canonical NF-κB pathway, and dysregulation of genes, including downregulated NF-κB target genes with known anti-tumor activity[37]. As mentioned before, cereblon is an essential drug target to mediate the anti-cancer effects induced by IMiDs. Transduced MM cells with shRNA for cereblon were less sensitive to lenalidomide compared to parental cells. Additionally, acquired resistance to IMiDs pomalidomide and lenalidomide was accompanied with a strong reduction in the cereblon protein. In 85% of lenalidomide refractory patients cereblon expression seemed to be decreased indicating its clinical relevance[253].

5.1.3 Alteration in drug metabolism

Systemic and intracellular drug concentrations are determined by drug metabolizing enzymes. Oxidation, reduction, hydrolysis and conjugation are crucial processes for the protection of normal cells against toxins. Unfortunately, these reactions are also able to induce drug resistance due to activation of prodrugs or increased inactivation of drugs. We previously illustrated a correlation between bortezomib resistance and the active Notch pathway. Activation of the Notch pathway induced the expression of a cytochrome P450 enzyme, CYP1A1, involved in drug metabolism. Blocking CYP1A1 rescued bortezomib sensitivity[315].

The response rate of diagnosed MM patients to induction therapy melphalan is situated between 70-80%, nonetheless all patients eventually relapse due to acquired resistance. Elevated gluthathion levels have been identified as one of the underlying mechanisms and this was linked with the upregulation of the y-glytamylcysteine synthetase enzyme. Blocking this last one with buthionine sulfoximine increased melphalan activity in MM xenograft models[316,317].

5.1.4 Clonal evolution and heterogeneity

Resistance and relapse of MM patients can also arise from an existing clone that has mutated or adapted to initial therapy (acquired resistance). For instance, the development of the different MM sub-clones with great genetic diversity during disease progression from MGUS to MM can lead to resistance[318]. They can differ in clonogenic potential, differentiation status and drug sensitivity. In high-risk patients it has been demonstrated that competing sub-clones can be present, evolve over time and develop therapeutic selection. Primary MM tumors can consist of different subpopulations such as B cells, activated B cells, pre-plasmablast cells, plasmablast cells and plasma cells as demonstrated by Leung-Hagesteijn et al[319,320]. Differences in bortezomib sensitivity could be observed for these different subpopulations and appeared to be linked to XBP-1 expression. Plasma cells and plasmablasts were XBP-1 positive and bortezomib sensitive (constituting the biggest population of the tumor) while pre-plasmablasts and earlier B cell progenitors (which are a minor but more immature population of the tumor) were negative and were insensitive to PIs. This population consequently expands and this results in relapse. MM patients resistant to bortezomib showed reduced expression levels of XBP-1 and IRE-1 compared to bortezomib sensitive patients. This clearly indicates that absence for XBP-1 promotes resistance to PIs and that tumor progenitors mediate treatment failure in MM[319,320].

Our lab investigated the clonogenic potential of $CD138^+$ and $CD138^-$MM cells. $CD138^+$ cells showed to have a higher tumor–initiating and clonogenic capacity compared to $CD138^-$MM cells. However $CD138^-$MM cells were less sensitive to PIs, IMiDs, dexamethasone and melphalan compared to $CD138^+$ cells[69].

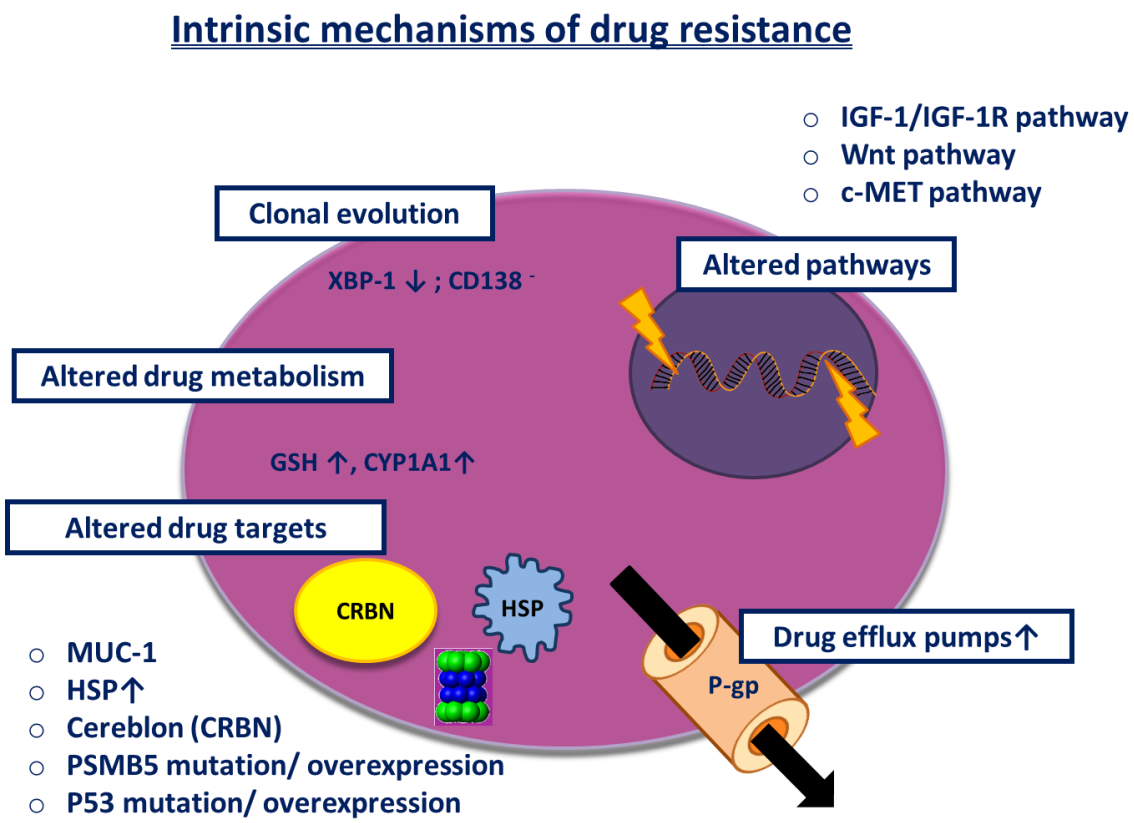

Figure 4: Summary of the different intrinsic mechanisms of drug resistance in multiple myeloma. *Mechanisms including increased drug efflux, clonal evolution, altered drug targets, drug metabolism and pathways are all implicated in the development of MM resistant cells to therapy. Mucin A C-terminal subunit (MUC-1); Heat shock protein (HSP); Glutathion (GSH); Cereblon (CRBN); Cytochrome P450 family 1, subfamily 1, polypeptide 1 (CYP1A1); insulin-like growth factor 1 receptor (IGF-1R). Adapted from* [321]

5.2 Extrinsic mechanisms

As already briefly mentioned before, BM microenvironment interactions are a crucial factor in MM pathology (section 2.4). Cell adhesion-mediated drug-resistance (CAM-DR) represents an extrinsic drug resistant mechanism caused by cell-cell contact or adhesion of cells to the extracellular matrix (ECM) proteins, including fibronectin. Adhesion of MM cells to fibronectin via β1 integrins results in a G1 arrest due to increased protein levels of p27kip1, inhibition of cyclin A and cyclin E kinase activity, upregulation of p21Cip1/Waf1 or downregulation of Bim (a member of the apoptotic Bcl-2 family)[89,92]. Antisense oligonucleotides for p27kip1 were not able to abrogate the adhesion of MM cells to fibronectin; however, MM cells became more sensitive to cytotoxic agents[89]. Furthermore, it has been demonstrated that β1 integrin adhesion increases IL-6 (gp130) mediated STAT3 phosphorylation in MM cells, leading to increased tumor proliferation and survival[322]. The sensitivity of MM cells to bortezomib and dexamethasone could be increased by the use of a monoclonal

antibody directed against IL-6, named CTNO 328[323]. These observations highlight the crucial role of IL-6 in anti-apoptosis and drug resistance in MM. Shain and colleagues demonstrated that adhesion of MM cells to fibronectin induced drug resistance by CD95-mediated programmed cell death by regulating the cellular localization and availability of c-FLIPL (c-Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein-long)[324]. Hepatocyte growth factor (HGF) promoted the adherence of MM cells to fibronectin via VLA-4 integrins and activation of the PI-3K/AKT pathway and NFκB pathway[325]. It has been observed that VLA-4 induces phosphorylation of several signal transduction molecules including CD19-receptor-associated protein tyrosine kinases and focal adhesion kinase (pp125FAK or FAK), which is an upstream activator of MAPK/ERK signaling pathway[326,327]. FAK plays a major role in inhibiting apoptosis both in adherent cells and suspension cells. Heat shock proteins (HSP) were also associated with CAM-DR as adhesion of MM cells to BMSCs and fibronectin upregulates HSP-70 in MM cells. Blocking HSP-70 resulted in enhanced melphalan-induced apoptosis[328]. Increased survivin expression could be observed after co-culturing human MM cell lines with BMSCs. Furthermore, inhibition of survivin reduced MM cell proliferation and increased sensitivity to doxorubicin, melphalan and dexamethasone[329]. Adherence of MM cells to BMSCs also increased B7-H1 molecules, Bcl-2 en FasL expression on MM cells and was associated with drug resistance to melphalan and dexamethasone[330]. TNFα secreted by plasma cells upregulates the expression of adhesion molecules on both MM plasma cells upregulates the expression of adhesion molecules on both MM cells and BMSCs, increasing the adhesion of both cell types to each other, with associated CAM-DR and induction of IL-6 and VEGF secretion[331].

Adhesion of MM cells to BMSCs induces activation of the Notch pathway. Pharmacological inhibition of the Notch pathway with a γ-secretase inhibitor (GSI) results in apoptosis of MM cells and prevented BMSCs-mediated drug resistance[332]. Treatment with GSI significantly increased the cytotoxicity of chemotherapeutic agents including doxorubicin and melphalan[332]. It has been found that lenalidomide-resistent cell lines overexpress the hyaluronan-binding protein CD44, a downstream target of the Wnt/β-catenin pathway[333]. Lenalidomide resistant models demonstrated a higher adhesion capacity and inhibition of CD44 by short hairpin RNA or monoclonal antibodies was able to reduce adhesion and increase sensitivity towards lenalidomide. Suppression of the Wnt/ β-catenin pathway by FH535 also increased lenalidomide cytotoxicity[333].

Chromosomal translocations and gene mutations could also be involved in CAM-DR induced drug resistance. For example, knockdown of MMSET protein in MM cells harboring t(4.14) reduced MM cell proliferation, induced apoptosis and changes two genes (DSG2 and ADAM9) that were associated with CAM-DR[59]. Mutations in oncogenes including RAS and c-MAF were also described to be involved in adhesion of MM cells to BMSCs[334,335].

6. Preclinical models in multiple myeloma

Preclinical *in vivo* models are essential and crucial tools for understanding the pathophysiology of MM. Over the years, different mouse models have been developed in order to study MM but also to improve current therapeutic approaches and to screen the efficacy of new treatments. Although, each model has his advantages, pitfalls and restrictions which will be briefly discussed in the next part.

6.1 5TMM models

The 5TMM models are syngeneic and immunocompetent murine models that were originally derived from aged C57Bl/KaLwRij mice that spontaneously developed MM. By isolation of plasma cells from the BM of diseased mice and subsequently intravenous injection in young healthy mice these models can be maintained[336,337] (Figure 5). Different models such as the 5T33MM, 5T2MM and 5TGM1 exist, these models are mimicking the human disease closely and are characterized by elevated M-protein levels in the serum, which are correlating with disease progression. Additionally, a major colonization of MM cells can be observed within the BM, spleen (5T2MM and 5T33MM) and the liver (5T33MM). The 5T33MM model is known to be aggressive and developing within 3 to 4 weeks, while the 5T2MM is a less aggressive model. This model takes 12 weeks to develop the disease. In contrast to the 5T33, the 5T2MM model also display occurrence of bone lesions[17,338,339]. The 5TGM1 model constitutes a sub-clone of the 5T33MM cells and can grow *in vitro* as well as *in vivo.* The characteristics of this model are similar to these of the 5T33MM model together with the occurrence of osteolysis[338,340]. The main asset of the 5TMM models is the close interaction with the syngeneic and immunocompetent BM environment in which they develop. This makes them attractive models to study the interactions between MM cells and the BM microenvironment, but also the intercommunication between different immune cells, such as NKT cells and MDSCs [341,342]. However, since these models are entirely murine, differences on biological aspects between mice and men can be a limiting factor for some experiment setups. Also in terms of genetics they only represent a single subtype of MM.

6.2 Transgenic models

Other models used to study MM are transgenic models. They are developed by dysregulating key genes involved in the MM development. The Eμ-XBP-1s and Eμ-MAF models for example, are using this approach where respectively x factor box binding protein-1 spliced isoform (XBP-1s) and MAF genes are dysregulated, leading to the formation of mixed B-lymphomas and MM-like tumors[343,344]. The Vk* MYC model, another preclinical murine model, is genetically engineered to activate the c-

myc oncogene under control of kappa light chain (Vk) regulatory elements. The activation of the MYC transgene in the germinal center of B-lymphocytes, results in the spontaneous development of MGUS, ultimately leading to MM[345]. These models are ideal to investigate the role of the dysregulated genes enhancing progression to MM, together with compounds targeting these genes. The pitfalls of these murine models hampering their general use are the labor intensiveness to generate them and the time consuming aspect regarding the development of MM.

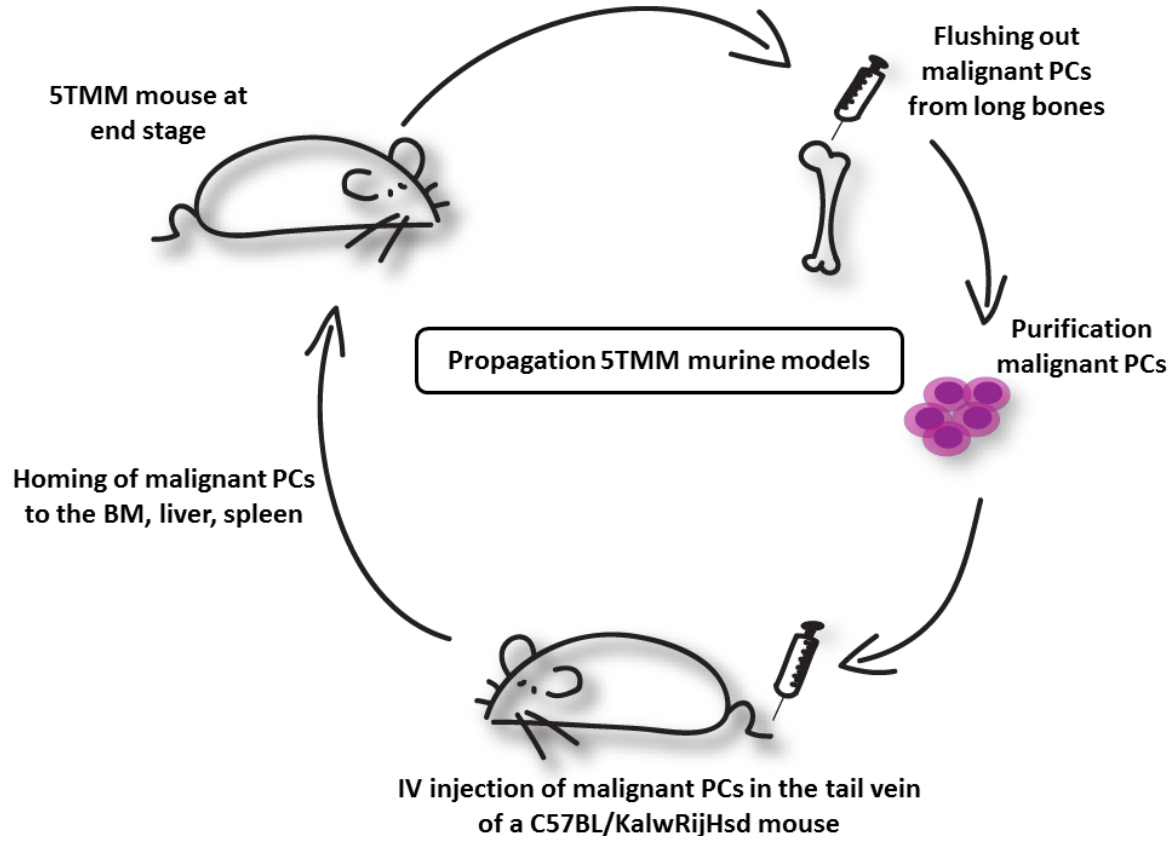

Figure 5: Schematic overview for the propagation and maintenance of the 5TMM murine models. PCs: plasma cells.

6.3 Xenograft models

In the xenograft models human MM cell lines or primary MM cells are engrafted into immunodeficient mice. These severe combined immunodeficient mice (SCID) lack functional B and T cells, preventing graft rejection. The engraftment of human MM cells can be administered locally or systemically resulting in palpable tumors or in a diffuse spread MM[346–348]. Nonetheless, the BM microenvironment is not taken into account and the success of engraftment rather remains poor.

Two other used engraftment strategies are based on the implantation of human fetal bones containing human myeloma cell lines or primary MM cells. Here, MM cell growth is sustained by the presence of the human fetal microenvironment[347,349,350]. However, some pitfalls are linked to this model such as ethical issues, the large number of primary MM cells needed as well as the allogeneic background of the BM microenvironment. By implantation of MM cells into three-dimensional bone-like poly-ε caprolactone polymeric scaffolds coated with murine or human BM stromal cells these limitations can be overcome[351]. Also ectopic MM models exists, where MM patient-derived BM biopsy are implanted into SCID mice. Upon growth, implants are removed and fragmented for serial passages by intramuscular/subcutaneous implantations or intravenous injections[352]. The intratibial injection of human MM cells is a relatively new model. MM cells seem to grow exclusively in the BM and major features of the human MM disease such as M-protein spike and bone lesions are exhibited in this model. The asset here is that primary MM cells obtained from newly-diagnosed patients with different classifications or from relapse refractory patients showing a resistant phenotype can be used[353]. In this way many models mimicking the patient heterogeneity can be developed when sufficient patient material can be obtained and if the engraftment was successful. The overall major limitations of xenograft models are the absent immune system and the fully compatible microenvironment. However, these models can be used to test novel potential anti-MM agents which are directly targeting the MM cells.

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Chapter II

The immunoregulatory role of iNKT cells in multiple myeloma

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Does an NKT cell-based immunotherapeutic approach have a future in multiple myeloma?

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Abstract

Natural killer T (NKT) cells constitute a unique subset of innate-like T lymphocytes which differ from conventional T cells by recognizing lipid antigens presented by the non-polymorphic major histocompatibility complex (MHC) I-like molecule CD1d. Despite being a relatively infrequent population of lymphocytes, NKT cells can respond rapidly upon activation with glycosphingolipids by production of cytokines which aim to polarize different axes of the immune system. Due to their dual effector capacities, NKT cells can play a vital role in cancer immunity, infection, inflammation and autoimmune diseases. It is believed that modulation of their activity towards immune activation can be a useful tool in anti-tumor immunotherapeutic strategies. Here we summarize the characteristics of NKT cells and discuss their involvement in immunosurveillance. Furthermore, an update is given about their role and the progress that has been made in the field of multiple myeloma (MM). Finally, some challenges are discussed that are currently hampering further progress.

1. Natural killer T cells: subsets and function

Natural killer T (NKT) cells constitute a highly conserved heterogeneous subset of innate-like T lymphocytes. This small population owns unique phenotypic and functional properties that set them apart from conventional T cells by exhibiting characteristics of both the innate and the adaptive immune system[1,2]. They diverge from conventional T cells by recognizing foreign and self (glyco)sphingolipid antigens presented by the non-polymorphic major histocompatibility complex (MHC) I-like molecule CD1d, expressed on professional antigen-presenting cells (APCs)[2,3]. Originally NKT cells were defined as expressing both the CD3 and αβ T cell receptors (TCR) and lineage markers from natural killer (NK) cells, such as CD56 or CD161 (human) and NK1.1 (murine). It is now generally accepted that this description is no longer accurate since these cells only seem to be a part of the broader NKT cell family[4]. Moreover, NKT cells have a remarkable capacity to produce extensive amounts of cytokines upon stimulation to activate NK cells, dendritic cells (DC), regulatory and conventional T cells and B cells[5,6]. Thereby enhancing a cascade of complementary cytokines and chemokines and stimulating additional populations to mediate immune surveillance[6]. Due to their broad cytokine profile, NKT cells can both exert an immune enhancing and immunosuppressive role and play therefore a vital role in various pathologies, such as cancer, infection, inflammation and autoimmune diseases[6–10]. Modulating their activity towards immune activation could be a useful tool for improving vaccines in cancer, infectious diseases and other therapeutic settings.

1.1 Type I Natural Killer T cells

The type I NKT cells also referred to as “invariant” NKT cells (iNKT) are the main studied subpopulation of NKT cells and are usually linked to promotion of tumor immunity. They express a semi-invariant TCRα chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) paired with a heterogeneous Vβ chain repertoire (Vβ2, 7 or 8.2 in mice and Vβ11 in humans)[2]. Type I NKT cells often express other NK surface markers such as NK1.1 (in some mouse strains) or CD161 (in human), NKG2D, CD44, CD56, CD69, CD94, CD122 and members of the Ly49 family[7,8]. iNKT cells can be further subdivided according to their CD4/CD8 co-receptor expression: $CD4^+$ and $CD4^-CD8^-$subsets, and a small subset of $CD8^+$ cells (only human) have been described[11–13]. Type I NKT cells are present in different tissues, such as the spleen (0,2 - 0,5% of the T lymphocytes), bone marrow, thymus, lymph nodes and blood (0,01 - 0,5% of the T lymphocytes) in mice[11,14]. The highest frequency is found in the liver with around 10 to 30% of all T lymphocytes[15]. These hepatic iNKT cells possess a strong anti-tumor capacity and show different functional characteristics than the NKT cells from other tissues[14]. In humans, type I NKT cells appear to be approximately 10 times less abundant in the liver than in mice while for the spleen, bone marrow, blood and lymph nodes the ranges remain similar. The highest prevalence is

found in the omentum, representing 10% of the white adipose tissue T cell population, whereas their frequency and number in the peripheral circulation vary widely between individuals[1,7,11,16]. Identifying lipid antigens recognized by NKT cells is still an ongoing challenge. Type I NKT respond to α-and β-linked glycosphingolipids among which α-Galactosylceramide (αGalCer, KRN700), an exogenous synthetic glycolipid originally extracted from the marine sponge Agelas mauritianus or microorganisms symbiotic with the sponge[4,17]. α-GalCer is the most-well characterized agonist for type I NKT cells in humans and mice and shows a very potent capacity to induce cytokine release by iNKT cells[2] . Today, several new analogues of αGalCer, showing weaker or stronger agonistic potential, have been synthetized including, α-C-GalCer, naphthylurea 6"-derived α-GalCer (NU-α-GalCer), C20:2, DB06-1 and OCH[18]. Also microbial and self-glycolipid iNKT cell antigens such as ceramide structures (Sphingomonas species), diacylglycerols (Borrelia burgdorferi and Streptococcus pneumoniae), cholesteryl-sugars (Helicobacter pylori) but also phospholipids (Mycobacterium tuberculosis), the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) and the peroxisomal derived lipid plasmalogen lysophosphatidylcholine (lyso-PC) have been identified[19–25]. More recently, in their quest to find new endogenous ligands Kain et al revealed the presence of mammalian α-linked glucosylceramides[26]. This defies the previous hypothesis where it was thought that humans were not able to make α-linked sugar moieties due to the presence of natural anti-α-linked sugar antibodies[27]. Direct CD1d-agonist stimulation of the TCR complex is accompanied by the rapid and robust release of T-helper 1 (Th1), Th2 and Th17 cytokines, including interferon-gamma (IFN-γ), interleukins (IL)-2, -4, -10, -13, -17, -21 and 22, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-alpha (TNFα)[3,7,10] (Figure 1). In addition, it was reported that cytolysis in a perforin-dependent manner, through the Fas-FasL axis or through expression of intracellular granzyme B is also promoted upon iNKT stimulation[28–30]. Similar to conventional T cells, through the engagement of costimulatory pathways such as CD40-CD40L and B7-CD28, DC are induced to maturate and secrete IL-12. In turn, IL-12 stimulates NK, NKT, and other T cells to produce IFN-γ which subsequently activates bystander cell activity and stimulates more downstream effector populations such as NK cells, $CD8^+$ T cells and γδ cells[7]. Type I NKT cells are indirectly activated in response to pattern-recognition receptor (PRR) of toll-like receptor (TLR) signalling by APCs together with presentation of self-antigens through CD1d, inducing cytokine secretion by APCs such as IL-12, IL-18 and type I α/β IFNs[7,21,31,32] (Figure 1). Also during inflammation, type I NKT cells can be stimulated in a TCR independent manner by different stimulatory and co-stimulatory signals, such as engagement of peroxisome proliferator activated receptor (PPAR)γ through bacterial products such as lipopolysaccharide (LPS)[31,33]. Activation of Fcγ receptors by antigen-IgG complexes, interaction of NK1.1 receptors with their ligands on APCs and activation of TLRs of previously activated type I NKT cells also take part in additional activation mechanisms of type I NKT cells[31].

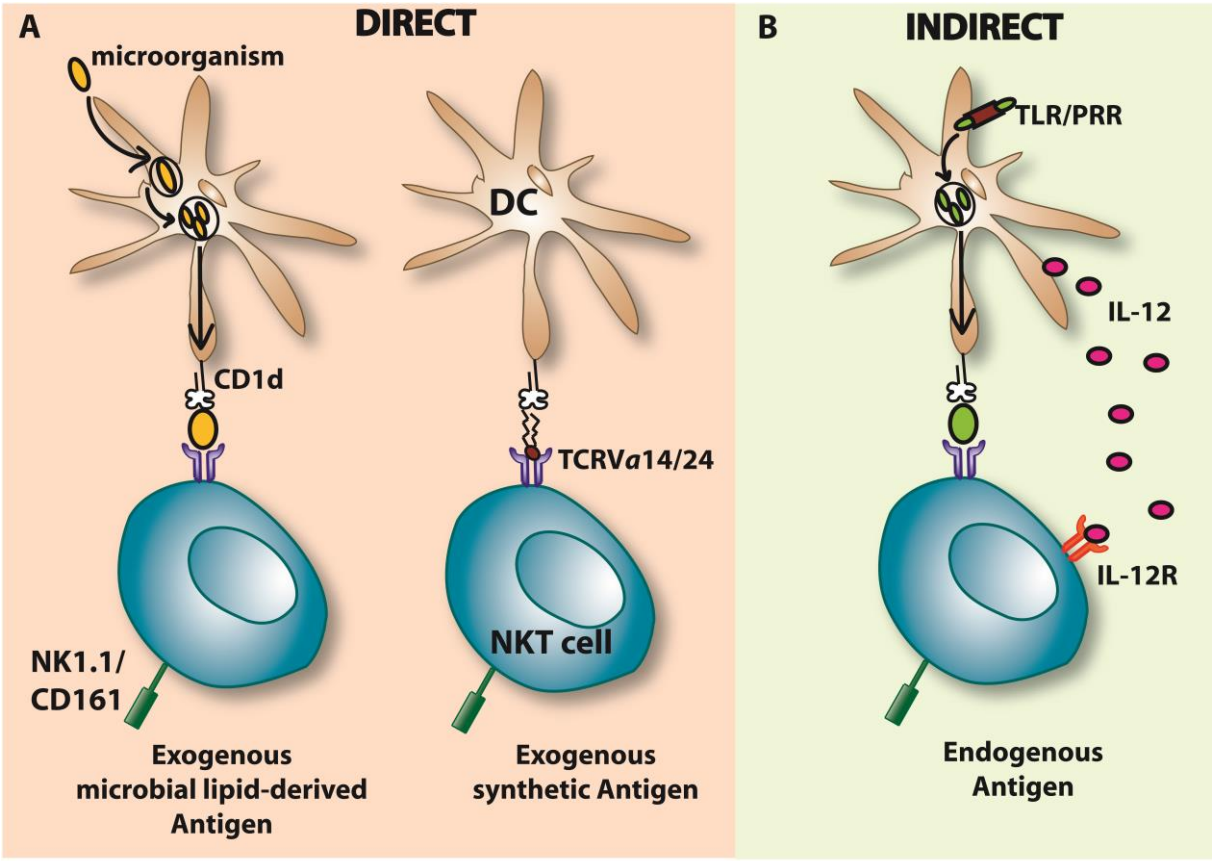

Figure 1: Major iNKT cell activation pathways. ***(A) Direct activation*** *of iNKT cells occurs when the TCRVα14/24 interacts with a ligand presented by a CD1d molecule present on DCs or other APCs. DCs present exogenous glycosphingolipids such as the synthetic α-GalCer or microbial lipid-derived antigens and subsequently activate the iNKT cell. This CD1d dependent activation is followed by the secretion of cytokines such as IFN-γ and IL-4.* ***(B) Indirect activation*** *of iNKT cells can be induced by cytokine secretion of DCs such as IL-12. Engagement of a microbial Ag to the pattern-recognition receptor (PRR) of the toll-like receptor (TLR) present on APCs (e.g DCs) triggers IL-12 co-stimulation. IL-12 secreted by DCs binds to its receptor, IL-12R, on iNKT cells which activates iNKT cells by inducing IFN-γ secretion. The activation occurs in the presence or absence of self or low affinity endogenous lipid antigens. Besides IL-12, also other cytokines such as IL-18 and type I-IFN (α & β) can be secreted and activate iNKT cells in a CD1d independent manner. iNKT, Invariant natural killer T; DCs, Dendritic cells; TCR, T cell receptor; α-GalCer, Alpha-Galactosylceramide; IL-12, Interleukin 12, IL-12R; Interleukin 12 receptor; TLR, Toll-like receptor; PRR, Pattern-recognition receptor; IFN, Interferon.*

1.2 Type II Natural Killer T cells

Type II NKT cells are a CD1d-restricted subset that expresses more diverse αβ-TCRs (for example Vα3.2Jα9 or Vα8 with Vβ8 TCRβ-chain). They have an activated or memory phenotype and many also express NK surface markers[3,34]. Moreover, they have the ability to suppress autoimmunity and inhibit tumor rejection. In contrast to type I NKT cells, their distribution and physiological role is less understood. They compromise a minor subset in mice but constitute a major subgroup of the T cells in the bone marrow, liver and gut of humans[15,35]. Type II NKT cells are non-reactive to α-GalCer.

Currently, the most widely studied type II NKT antigen is sulfatide, a glycolipid abundantly present in neuronal tissue, liver, kidney and pancreas[36,37]. Recent research also identified a range of hydrophobic antigens, such as lysosulfatide, lyso-PC, small aromatic (non-lipid) molecules and other lipids such as β-Glucosylceramide (β-GlcCer)(C24:0) and β-Galactosylceramide (β-GalCer) as being potential activators of type II NKT cells[38–41].

1.3 Other invariant-like T cells

Next to the different subsets of NKT cells, it is worth to briefly mention another population of semi-invariant T cells, called mucosal-associated invariant T cells or MAIT cells. They are restricted to a monomorphic MHC I-like molecule MR1. Similar to NKT cells, they express an invariant TCRα-chain (Vα33Jα19 in mice and Vα7.2α19 in humans) combined with a limited but not invariant range of TCRβ-chains[42]. Rare in laboratory mice, they appear to be a very significant subset of T cells in humans, accounting for 1-10% of T cells in peripheral blood and being predominantly present in liver and mucosal tissues. Surprisingly, a completely new and unexpected class of antigens was shown to be presented by MRI molecules to MAIT cells, namely vitamin B2 (riboflavin) metabolites[43–45]. Although these cells are not CD1d restricted, their similarities with NKT cells are intriguing[46]. Research on MAIT cells has till now been hampered due to the lack of identification tools and the unknown nature of the antigens. However, very recently Reantragoon et al. were able to develop MR1 tetramers which allowed them to better phenotypically characterize human and mouse MAIT cells[47]. This development will lead to an increased understanding of the nature of MAIT cells. Also γδ T cells belong to this non-conventional invariant T cell group. Being innate-like lymphocytes, they differ from conventional αβ T cells since they do not express the CD4 and CD8 co-receptors but express Toll-like receptors and share a number of markers with NK cells[48]. Subsequently, antigen recognition by γδ TCR is not restricted to MHC molecules. γδ TCR recognize a diverse array of self and nonself-antigens, such as small peptides, soluble or membrane proteins, phospholipids, prenyl pyrophosphates, and sulfatides, while αβTCR bind peptides presented by MHC class I or class II molecules. In humans, γδ T cells represent 0.5 – 16% (on average: 4%) of all CD3+ cells in adult peripheral blood, and organized lymphoid tissues (thymus, tonsil, lymph nodes, and spleen), <5% in tongue and reproductive tract and 10 – 30% in intestine. In adult mice, 1 – 4% of all T cells in thymus, secondary lymphoid organs and lung are γδ T cells. γδ T cells are more abundant in other mucosal sites where they constitute 10 – 20% of all T cells in female reproductive organs, 20 – 40% of the intestinal intra-epithelial T cells and 50 – 70% of skin dermal T cells[49]. Moreover, the γδ TCR repertoire is restricted and depends on the tissue type and their localization. The conditions that lead

to responses of γδ T cells are not fully understood, and current concepts of them are 'first line of defense', 'regulatory cells', or 'bridge between innate and adaptive responses'.

2. Natural killer T cells: Implications in tumor immunity

2.1 Enhancement of tumor immunity

Type I NKT cells are able to kill cancer cells directly or indirectly via the downstream activation of other innate and adaptive immune cells. Direct NKT lysis can be induced by perforin , via the Fas-FasL axis or through expression of intracellular granzyme B[28,50]. *In vitro* observations demonstrated that tumor cells expressing CD1d were more prone to lysis induced by NKT cells[51,52]. This strengthens the hypothesis that high CD1d expression levels on tumor cells correlate with lower metastasis rates[52]. However, most of the tumor immunosurveillance by type I NKT cells is initiated by Th1 cytokines and is mainly dependent on the recruitment and activation of other cytolytic cell populations. In fact, large amounts of IFN-γ and cross-activation of NK cells are necessary for tumor protection upon α-GalCer stimulation. Cytokines such as IL-12 and IL-18 are also necessary to reach optimal IFN-γ levels, consequently leading to tumor immunity[53–55]. Proof that tumor immunosurveillance by type I NKT cells occurs through CD1d became clear when adoptive transfer of liver DN type I NKT cells from WT into CD1d KO mice (lacking all NKT cells) did not confer protection. In Jα18 KO mice (missing type I but retain type II NKT cells) the NKT cell population was able to be recovered and tumor immunity could be rescued upon NKT cell transfer[30,56]. Nevertheless, in contrast with $CD4^+$ liver type I NKT cells, protection could only be generated using the DN liver type I NKT subset. From these studies it can be concluded that different subsets of NKT cells can have different functions in tumor immunosurveillance[14]. Surface marker expression, anatomical origin as well as different antigens can alter the immunological capacity and function of NKT cells. Type I NKT cells not only increase protective cell responses but can also enhance tumor immunity by modifying the effects of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), suppressive IL-10 producing neutrophils and T regulatory cells[57–60].

2.2 Suppression of tumor immunity

Type II NKT cells possess an immunosuppressive activity in tumor immunology. By counteracting type I NKT cells and negatively influencing other immune cells they are capable to down-regulate tumor immunosurveillance[61,62]. $CD4^+$ type II NKT cells are producing more IL-13 and IL-4 than type I cells[63]. By the release of Th2 cytokines, type II NKT cells have been shown to suppress autoimmune T cell responses. The original observation was made in a 15-12RM fibrosarcoma model where $CD8^+$

cytotoxic T cells were suppressed by $CD4^+$ type II NKT cells through production of IL-13 which in turn induced TGF-β, leading to suppression of the anti-tumor activity[63,64]. Later on, a similar observation was also reported in several other solid tumor models such as in a CT26 colon carcinoma lung metastasis model, a subcutaneous CT26-L5 colon carcinoma model, an orthothopic K7M2 osteosarcoma model and a renal cell adenocarcinoma liver metastasis model[65]. CD1d KO mice and Jα18 KO mice were compared side-by-side in different tumor models. CD1d KO mice were resistant to tumor growth while Jα18 KO mice behaved similar to wild type mice. This confirms the hypothesis that type II NKT cells present in Jα18 KO were sufficient for suppression of tumor immunosurveillance. Anti-CD4 treatment was able to abrogate the retained suppression, consistent with the original observation that the suppressing cell type has a $CD4^+$ phenotype[65]. Furthermore, direct selective stimulation by sulfatide significantly induced growth of CT26 lung metastasis. The effect was retained in Jα18 KO mice but was lacking in CD1d KO mice. This indicated that the effect of sulfatide was only type II NKT cell specific. As a result, it was assumed that type II NKT cells also suppress anti-tumor immune responses in humans in a similar way[61].

Although the immunosuppressive role is often attributed to type II NKT cells, there are a number of exceptions reported in literature where type I NKT cells appear to support immunosuppression[66–68]. Th2 cytokines (IL-13, TGF-β) produced by type I NKT cells conferred immunosuppression, subsequently leading to the inhibition of cytotoxic T cells and NK cell activity. The outcome of type I NKT cell-activation is dependent on different factors such as the antigens, co-stimulatory signals and the cytokine milieu which determine the plasticity of these cells. Immunosuppressive Tregs have been shown to be supported by activated type I NKT cells through IL-2 production, but subsequently suppressed the NKT cells in a cell-cell contact manner[69]. Two studies have reported type I NKT cells capable of directly suppressing tumor immunity in animal models of hematological malignancies. In a RMA/ T cell lymphoma model, NKT deficient mice had augmented cytotoxic T cell activity and greater survival rates than WT mice[67]. In a model of Burkitt's-like B cell lymphoma, Jα18 KO mice had significantly fewer splenic tumors than WT or CD1d KO mice. Stimulation of type I NKT cells with α-GalCer did not increase tumor burden, it decreased tumor specific $CD8^+$ T cells[66].

2.3 Cross regulation

It has been demonstrated that type I and type II NKT subsets not only exert positive or negative effects on different cell populations but also cross-regulate each other. CD1d KO mice, deficient in both NKT types, showed strong resistance towards tumor growth in the CT26 colon carcinoma model, whereas Jα18 KO mice, lacking only type I NKT cells, showed higher sensitivity to tumor growth than WT mice. Consequently, this suggests that type I NKT cells may reduce the suppressive

effect of type II NKT cells[61]. The *in vivo* activation of type II NKT cells by sulfatide enhanced new tumor formation, and abrogated or reduced the positive clinical effects of α-GalCer when administered together. For example, decreased pro-inflammatory cytokine secretion was observed, thereby indicating that type II NKT cells may also have the ability to suppress type I NKT cell activation. Addition of the type II antigen sulfatide *in vitro* inhibited α-GalCer-induced IFN-γ, IL-2, and IL-4 production[61,63,70,71]. Moreover, Halder RC et al demonstrated by use of the model of concanavalin A-induced hepatitis, that the activation of sulfatide-reactive type II NKT cells and plasmacytoid DCs in the liver contributed to the anergy, or hyporesponsiveness of type I NKT cells[72]. However, it is unclear if this mechanism is similar in a tumor setting. Nevertheless, the finding that immune-protective type I and immune-suppressive type II NKT cells cross-regulate each other establishes an NKT regulatory axis and creates the opportunity to exploit this knowledge in a clinical setting. The success of immunotherapies may depend on which way the balance of the axis is shifted. Enhancing the activity of type I NKT cells while simultaneously blocking type II NKT cells could be a promising strategy for future anti-tumor therapies[73].

3. Natural killer T cells: Implications in multiple myeloma

Different research groups provided evidence of type I NKT cells being quantitatively and qualitatively defective in MM, subsequently hampering their anti-tumor effects[74] (Figure 2). The group of Dhodapkar et al. demonstrated that type I NKT cells are still detectable in the blood and tumor bed of MM patients at both early and progressive stages of the disease but they could observe that advanced stages of MM were associated with the progressive loss of the ability of iNKT cells to secrete IFN-γ. The type I NKT dysfunction could be overcome *in vitro* by using dendritic cells (DCs) pulsed with α-GalCer. When MM patients were injected with α-GalCer loaded DCs (at monthly interval 2 injections), their type I NKT cell pool expanded 100 fold, with improved function and these effects lasted for several months. Altogether, these results suggest that clinical progression is linked to an acquired but potentially reversible defect in type I NKT cells and supports the hypothesis that iNKT cells help in controlling the malignant growth of the MM cells[74,75]. Together with other groups they further demonstrated that MM cells are expressing CD1d and are sensitive to lysis induced by type I NKT cells, making them interesting targets for NKT directed therapies[74]. Spanoudakis et al has shown that CD1d was highly expressed on premalignant and early myeloma. With disease progression CD1d expression levels were down-regulated and eventually lost altogether in advanced MM patients and in most of the studied myeloma cell lines, leading to a reduction in survival[76]. Engagement of CD1d by anti-CD1d monoclonal antibodies was able to induce myeloma cell death *in*

vitro which was not induced by caspase-activation but was rather associated with overexpression of the pro-apoptotic protein Bax and mitochondrial membrane potential loss[76].

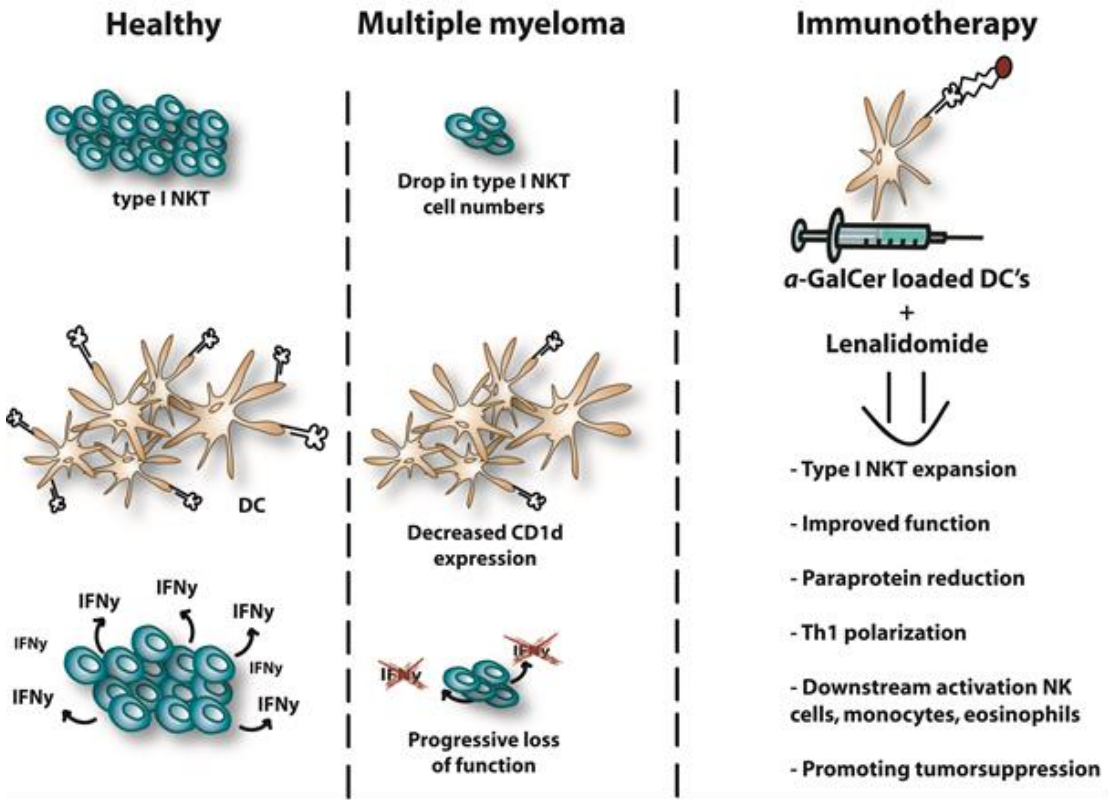

Figure 2: NKT dysregulation in multiple myeloma. *Type I NKT cells are quantitatively and qualitatively defective in MM which hampers their anti-tumor potential. A dramatic drop of type I NKT cell number can be observed in tumor mouse models and MM patients. CD1d expression levels are down-regulated in advanced MM patients and expression is lost in most of the studied myeloma cell lines. Moreover, advanced stages of MM are associated with a progressive loss of function and their capacity to secrete IFN-γ. LEN has the ability to induce type I NKT expansion in presence of α-GalCer and to stimulate IFN-γ secretion by NKT cells in MM patients. Combination therapy provides downstream activation of NK cells, monocytes and eosinophils and ultimately promotes tumor suppression. A reduction of paraprotein is detected in the serum or urine. NKT, Natural killer T; DC, dendritic cells; NK, natural killer; Th, T helper; α-GalCer, Alpha-Galactosylceramide; IFN, Interferon.*

We recently investigated the number, activity and characteristics of type I NKT cells in the syngeneic preclinical 5T33MM murine model, an immunocompetent model which mimics the human disease closely[77]. Consistent with previous observations, our results demonstrated a dramatic drop of type I NKT cell numbers in the liver and spleen at the end stage of the disease. This decline was also detectable in the 5T2MM model, a slower progressing model. The ability of murine type I NKT cells to secrete IFN-γ in response to α-GalCer loaded mature DCs was abrogated at the end stage of the disease due to a decline in NKT number. Treatment with α-GalCer loaded DCs significantly increased

the survival of MM diseased mice for 1 week when they were injected on the same day of 5T33MM inoculation[77]. The group of Mattarollo et al. could also demonstrate that a single vaccination of irradiated tumor cells pulsed with α-GalCer was able to inhibit MM development and prolong survival of Vk*MYC mice[78]. Nonetheless, the expression of CD1d in the 5T33MM model was still high at the end stage of MM and lacked the potency to activate type I NKT cells and cause tumor cell lysis after stimulation with α-GalCer. We also found that the 5T33MM cells lacked the necessary co-stimulatory molecules such as CD40, CD80 and CD86 potentially explaining our observations[77]. Hong et al. however demonstrated that a vaccine consisting of α-GalCer-loaded MOPC315BM myeloma cells efficiently promoted anti-tumor immunity, slowed down tumor growth, induced established tumor regression and protected (surviving) mice from tumor rechallenge. Strong humoral immune responses, including myeloma-specific antibodies and cellular immune responses, such as myeloma-specific $CD8^+$ cytotoxic and memory T cells were induced and Treg cells were significantly decreased[79]. It is known that MM correlates with a high vascular index. Targeting angiogenesis is therefore an important therapeutic tool to reduce MM progression. We were able to demonstrate that the conditioned medium of α-GalCer stimulated NKT cells induced a reduction in endothelial cell proliferation, migration and network formation and increased their apoptosis *in vitro*, whereby the JAK-STAT signalling pathway was highly activated. Furthermore, injecting α-GalCer *in vivo* led to a significant reduction in microvessel density[80].

Song et al. succeeded in activating and expanding CD1d-restricted type I NKT cell lines isolated from newly diagnosed and advanced MM patients[81]. The results showed that type I NKT cells could secrete Th1-polarized cytokines in response to α-GalCer loaded DCs or primary MM cells and that they could induce direct cytotoxicity against the primary MM cells. LEN, a derivate of thalidomide and one of the novel drugs used to treat MM, is effective in inducing complete or good partial responses and is able to improve the survival of MM patients[74]. It has among others immunomodulatory properties, although the specific cellular targets and molecular mechanisms responsible for the immunomodulatory actions of LEN have not been fully elucidated yet. Song et al. provided preclinical evidence that a combination of type I NKT immunotherapy with LEN led to an increased Th1 cytokine production and reduced Th2 cytokine levels (Figure 2)[81]. The group of Chang et al. observed an even greater effect when LEN was combined with dexamethasone[75]. They further obtained striking results when α-GalCer loaded DCs were injected in 3 MM patients at stage III. Intravenous injection of α-GalCer loaded mature DCs in these patients, who had received chemotherapy and stem cell transplantation, gave a remarkable boost in the expansion of circulating type I NKT cells which sequentially resulted in a reduction of the serum and urine levels of M-protein. A sustained expansion of type I NKT cells, lasting 3 months after vaccination, was observed in one of the patients.

An increase of different factors such as IL-12 p40, IP-10 and MIP-1β in the patient serum levels were detected[75]. Confirming previous *in vitro* results, Dhodapkar et al found that LEN had the ability to enhance type I NKT expansion in presence of α-GalCer and to stimulate IFN-γ secretion by NKT cells in both healthy donors and MM patients (Figure 2). The combination therapy provided downstream activation of NK cells, monocytes and eosinophils by upregulating surface receptors such as NKG2D, CD56 and CD16, ultimately promoting tumor suppression[82].

Data on type II NKT cells and MM are scarcely present in literature. However, their increase in the peripheral blood of MM patients was reported by Chang DH et al. Those type II NKT cells appeared to be specific for lyso-PC and had a Th2-skewed profile with high expression levels of IL-13[39]. Taken together, these data suggest that NKT cells are a particularly attractive subset to target and encourage the rationale for type I NKT cell-mediated immunotherapy in MM.

Tabel 1. Brief overview of α-GalCer–based clinical trials in different cancers.

Therapeutic setting	Cancer type	Clinical outcome	Immunological responses	Ref.
α-GalCer (i.v.)	Solid tumors	7 out of 24 patients had stable disease	Increase in IL-12, GM-CSF and TNF-α, serum levels	83
α-GalCer-pulsed immature MoDCs (i.v. & i.d.)	metastatic malignancies	2 patients out of 12 with decreased tumor markers in serum, 1 with tumor necrosis	Expansion NKT cells, activation of T and NK cells, increased IFN-γ levels	29
α-GalCer-pulsed immature MoDCs (i.v)	Non-small cell lung cancer	5 out of 9 had no change in disease status, 4 patients had disease progression, but 1 case had increase in NKT cells, 2 cases had significant responses	Expansion NKT cells, increase in IFN-γ mRNA levels	84
α-GalCer-pulsed mature MoDCs (i.v)	Anal cancer Renal cell cancer Multiple myeloma	The patient had stable disease The patient had stable disease The 3 patients had decreased levels of paraprotein in serum or urine	Expansion NKT cells and antigen-specific memory T cells	75
***Ex vivo* expanded NKT cells with autologous α-GalCer-pulsed PBMCs (i.v.)**	Non-small cell lung cancer	4 out of 6 patients had a stable disease, 2 patients had disease progression	Expansion NKT cells, elevated IFN-γ cell number	85
α-GalCer-pulsed autologous APCs (via nasal submucosa)	Head and neck squamous cell carcinoma	1 out of 9 patient had a partial response, 5 patients had a stable disease, 3 patients had disease progression	Expansion NKT cells, elevated IFN-γ cell number in tumor tissue and PBMCs	86
α-GalCer-pulsed APCs (i.v.)	Non-small cell lung cancer	5 out of 17 patients had a stable disease, the remaining 12 had disease progression	Expansion NKT cells, elevated IFN-γ cell number in tumor tissue and PBMCs	87
***In vitro* expanded NKT cells (i.a.) and α-GalCer-pulsed APCs (via nasal submucosa)**	Head and neck squamous cell carcinoma	1 out of 8 patients had disease progression, 3 patients reacted partially, 4 patients had a stable disease	Expansion NKT cells, elevated IFN-γ cell number in tumor tissue and PBMCs	88
***In vitro* expanded NKT cells (i.a.) and α-GalCer-pulsed APCs (via nasal submucosa)**	Head and neck squamous cell carcinoma	5 out of 10 patients reacted partially, 5 patients had a stable disease	Expansion NKT cells, elevated IFN-γ cell number in tumor tissue and PBMCs	89
α-GalCer-pulsed mature MoDCs (i.v) and LEN (oral, 10mg/day, 3 28 day cycles)	Multiple myeloma	3 out of 6 patients with decreased levels of paraprotein in serum or urine	NKT, NK, monocyte and eosinophil activation	82

α-GalCer: alpha-galactosylceramide; APCs: antigen-presenting cells; GM-CSF: granulocyte macrophage colony-stimulating factor; i.a.: intraarterial; i.d.:intradermal; i.v.: intravenous; MoDCs: monocyte-derived dendritic cells; NKT: natural killer T cells; PBMCs: peripheral blood mononuclear cells; TNF-α: tumor necrosis factor-alpha; LEN: lenalidomide

4. Natural killer T cells: Challenges

The use of α-GalCer and other glycolipids to activate type I NKT cells has engendered a lot of preclinical success in mice, leading to multiple clinical trials in humans (Table 1). However, the benefits for patients remains limited since the translation of these preclinical benefits into clinical trials is associated with some challenges[90]. As noted above, the frequency of type I NKT cells is much lower in humans than in mice and numbers are more variable between individuals which possibly can contribute to the heterogeneity in clinical responses[15,91,92]. In advanced stages of cancers, like MM, the number and function of type I NKT cells is often reduced. Therefore, the effects of NKT activation may be less amplified in humans than in mice[90]. Moreover, we can also presume that patients that participated in these trials had a much more advanced disease than mice in which α-GalCer had a significant greater therapeutic effect. It is worth to mention that it has been demonstrated in mice that following injection of α-GalCer, type I NKT cells cannot be restimulated for at least two month[93–95]. This means they are sensitive to anergy which is a property that can also explain the lack of success in humans. Being confronted in our research with this problem as well we have the opinion that this is one of the major obstacles which need to be overcome to give a future to an NKTbased immunotherapeutic approach. Specifically, marked increase in programmed death-1 (PD-1) expression after α-GalCer stimulation has been shown to hamper the beneficial and/ or long lasting effects of NKT cell-mediated treatment[96]. We also believe that a suboptimal activation due to uncontrolled distribution of α-GalCer remains a big problem. Therefore, it would be of great value to develop new α-GalCer carrier systems (e.g. nanovectors, liposomes and exosomes) to optimize NKT cell responses and cancer immunotherapy[97,98]. Also the different (sub)populations, their adaptable reactivity against ligand agonists and the different APCs involved in the antigen presentation add more complexity and can explain the paradox regarding the NKT cell subpopulations. Furthermore, it is possible that the presence of different endogenous self-antigens, leading to auto-reactivity, can activate different pathways in the NKTs that are modulating the NKT cell – cell talk[99]. Finally, our knowledge of the presence of endogenous ligands is still very limited hampering our true understanding of NKT cell biology[26].

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Chapter III

Aims of the PhD project

In multiple myeloma (MM), survival and progression largely rely on the crosstalk with the bone marrow (BM) microenvironment, inducing immune escape, angiogenesis, bone destruction and drug resistance. Despite great therapeutic advances, still most of the MM patients relapse and remain incurable. Over the past years, immunotherapy has emerged as a new field in cancer therapy. Here, the immune cells of the patient itself are activated to target the tumor. In MM, several effector cells of the immune system are present in the BM microenvironment; unfortunately, they are mostly all functionally impaired. In this study we focused on the immune system in MM, particularly on CD1d-restricted T cells recognizing glycosphingolipid-based antigens, the invariant natural killer T (iNKT) cells. These cells have the capacity upon activation to rapidly release copious amounts of cytokines, such as interferon (IFN)-γ, affecting a wide range of innate and adaptive immune responses. In recent years, manipulation of iNKT cells emerged as an attractive target for immunotherapy due to their crucial role in the initial steps of protective and/or anti-tumor immunity. The discovery of synthetic compounds such as α-Galactosylceramide (α-GalCer), potently activating iNKT cells, led already to a better manipulation of their immune responses. We and others demonstrated iNKT cell defects in MM, including impaired IFN-γ production, decreased cell prevalence and a strong reduction in CD1d expression levels on antigen presenting cells. While the total picture of iNKT cells as players in the immune system is rapidly evolving, the cause of their deficiency in hematological malignancies, such as MM, still remains to be determined. Therefore this study aimed to unravel and overcome mechanisms being responsible for iNKT cell deficiency in MM by use of patient samples and the preclinical immunocompetent 5T33MM murine model.

Specific aims of the study:

1. To investigate the crosstalk between leptin receptor activation and iNKT cell-mediated anti-tumor immunity in MM development.

Fatty deposits can occupy up to 70% of the BM cavity with aging, yet surprisingly little attention has been given to the role of BM adipocytes and their secreted adipokines in MM development. BM adipocytes may support MM oncogenesis by stimulating migration and proliferation of malignant plasma cells through secretion of myeloma-supportive adipokines such as leptin and are known to be negative regulators of the hematopoietic environment. Leptin plays a crucial role in energy homeostasis but also displays immune modulatory properties. Nevertheless, its role in anti-tumor immunity is unclear. iNKT cells have been shown to closely interact with adipocytes in a CD1d dependent manner. Because increasing fat deposition with aging coincides with elevated risk for MM development, we investigated if this could be the result of an impaired iNKT cell anti-tumor immunity mediated by adipokines, such as leptin (Chapter IV).

2. To investigate the role of the immune checkpoint molecule, PD-1, in iNKT and Mucosal-associated invariant T (MAIT) cell deficiency in MM.

MM cells create an immunologic milieu characterized by the disruption of effective antigen presentation, loss of effector cell function and complexity, and upregulation of pathways that promote tolerance and T cell anergy. A critical element of tumor-mediated immunosuppression is the presence of the negative checkpoint molecules, such as PD-1, that inhibit immune activation and the expansion of antigen specific T cells. In health, these pathways represent an essential stopgap against hyperactivation and the generation of autoimmunity. In the setting of malignancy, upregulation results in an exhausted T cell phenotype that promotes disease growth and resistance to immunotherapy. Therefore, we aimed to evaluate the implications of PD-1 in invariant T cell deficiency, i.e. iNKT and MAIT cells (Chapter V).

Chapter IV

Leptin receptor antagonism of iNKT cell function: a novel strategy to combat multiple myeloma

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Leptin receptor antagonism of iNKT cell function: a novel strategy to combat multiple myeloma

Leptin receptor blockade in myeloma

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Abstract

A hallmark of bone marrow changes with aging is the increase in adipocyte composition, but how this impacts development of multiple myeloma (MM) is unknown. Here, we report the role of the adipokine leptin as master regulator of anti-myeloma tumor immunity by modulating the invariant natural killer T (iNKT) cell function. A marked increase in serum leptin levels and leptin receptor (LR) expression on iNKT cells in MM patients and the 5T33 murine MM model was observed. MM cells and leptin synergistically counteracted anti-tumor functionality of both murine and human iNKT cells. *In vivo* blockade of LR signalling combined with iNKT stimulation resulted in superior anti-tumor protection. This was linked to persistent IFN-γ secretion upon repeated iNKT cell stimulation and a restoration of the dynamic antigen-induced motility arrest as observed by intravital microscopy, thereby showing alleviation of iNKT cell anergy. Overall our data reveal the LR axis as novel therapeutic target for checkpoint inhibition to treat MM.

Introduction

Multiple myeloma (MM) is a monoclonal plasma cell cancer predominantly developing in the bone marrow (BM). Despite great improvements with novel therapeutic agents achieved in the past decade, MM still remains incurable for the majority of patients. Therefore, there is a unmet need for novel therapeutic targets[1].

In MM, a complex crosstalk of different cell types within BM confers survival and growth to MM cells and induces angiogenesis, bone destruction, immune escape and drug resistance[2]. Fatty deposits occupy up to 70% of BM cavity with aging, yet surprisingly little attention has been given to the role of BM adipocytes and associated adipokines in MM development. BM adipocytes may contribute to MM oncogenesis by promoting migration and proliferation of malignant cells through secretion of myeloma-supportive adipokines such as leptin. Moreover, they are known to be negative regulators of the hematopoietic environment[3,4]. Recently, it became clear that adipose tissue itself contains a broad range of immune cells, both of the innate as well as the adaptive origin. Reciprocal interactions of immune mediators with adipocytes have been described, giving rise to the emerging field of immunometabolism[5]. Invariant natural killer T (iNKT) cells, are unconventional, lipid antigen reactive T cells with important roles in anti-tumor immunity and have been shown to closely interact with adipocytes in a CD1d dependent manner [6–8]. In MM, defective IFN-γ production by iNKT cells and reduced iNKT cell frequencies were described [8–12]. Moreover, CD1d expression levels on antigen presenting cells are progressively downregulated upon MM progression[8,13]. Because increasing fat deposition with aging coincides with elevated risk for MM development, we hypothesized this could be the result of impaired anti-tumor immunity mediated by leptin. We therefore tested the crosstalk between BM adipose tissue and iNKT cell function through release of the leptin in the onset of MM.

Material and methods

Mice, cell lines, flow cytometry and antibodies

Details of the 5T33MM murine model, C57BL/KaLwRijHsd CD1d$^{-/-}$mice, cell lines, flow cytometry and antibodies used in this study are presented in supplementary materials and methods.

Myeloma patients

BM, peripheral blood and serum samples were collected from untreated newly diagnosed MM patients (NDMM, n=19) and from age and gender matched healthy controls (n=11) and MGUS patients (n=18). Patient characteristics are summarized in supplementary Table 1. All patients gave informed consent, and the study was conducted in accordance with the Declaration of Helsinki. Approval was obtained by the Ethic Board of UZ Brussel (B.U.N. 143201316382) and Tumourbank of Lille (CSTMT102).

iNKT – MM cell co-culture assay

For murine experiments, isolation and expansion of 5T33MMvv, 5T2MM cells and iNKT cells were performed as previously reported[14–16 17]. The isolation of DCs and loading with α-GalCer is explained in the supplementary materials and methods as well as the iNKT cell expansion. 5x10^4 5T33MMvv / 5T33MMvt / 5T2MM cells, 5x10^4 iNKT cells, 10^5 α-GalCer loaded or vehicle loaded DCs were co-cultured in triplicate. Murine recombinant leptin (0,25 µg/ml, Sigma-Aldrich) and/or bispecific nanobody 2.17-mAlb (50 µg/ml) were added. Construction, production, and purification of nanobody 2.17-mAlb is described elsewhere[18]. Supernatants of co-cultures were collected after 48 h for IFN-γ measurements by ELISA (eBioscience).

For human co-culture experiments, iNKT cells were expanded by isolating PBMCs from healthy adult individuals using Ficoll centrifugation and incubating them for 7 days with α-GalCer (100ng/ml) and IL-2 (5U/ml, Roche). Subsequently, PBMCs were incubated with mAbs directed against TCRVβ11, TCRVα24 (Beckman Coulter) and 7-AAD (Becton Dickinson). TCRVα24$^+$Vβ11$^+$7-AAD$^-$(iNKT fraction) were cell sorted into 96-well plates (FACSAria, Becton Dickinson). TCRVα24$^-$Vβ11$^-$7-AAD$^-$CD3$^+$ cells were used as iNKT depleted T cells and stimulated with anti-CD3 and anti-CD28 Abs (eBioscience). Antigen presenting cells (APCs, 1x10^5) were collected from PBMCs after CD2 Dynabeads depletion (Invitrogen), loaded with α-GalCer (100ng/ml) or vehicle and subsequently co-cultured with 5x10^4 RPMI 8226/ JJN-3/ U-266 MM cells and 5x10^4 iNKT cells. Human recombinant leptin (0,25 µg/ml, R&D Systems) and/or bispecific nanobody 2.17-mAlb (50µg/ml) were added. Also for stimulated

iNKTdepleted T cells, effects of recombinant leptin and/ or RPMI 8226/ JJN-3/ U-266 MM cells were tested similarly as described above. Supernatants of co-cultures were collected after 24 h for IFN-γ measurements by ELISA (eBioscience).

***In vivo* treatment with α-GalCer and 2.17m-Alb leptin receptor antagonist in MM**

C57BL/KaLwRijHsd and C57BL/KaLwRijHsd CD1d$^{-/-}$mice were randomized and inoculated with or without 5T33MM as previously described and weekly i.p. injected with 2µg α-GalCer[14,19]. Serum IFN-γ levels were determined by ELISA after the primary and second stimulation with α-GalCer. Mice were treated for 10 days with 2.17-mAlb or control BcII10-mAlb nanobody (i.p., 200 µg mouse^{-1} day^{-1}) and weighted daily[18,20]. After α-GalCer restimulation flow cytometry was performed on 3 mice per group to determine activation, BrdU incorporation and intracellular cytokine release of iNKT and NK cells in indicated organs. At early established disease monitored and identified by two independent researchers, serum M-spike levels, TNFα and IL-6 levels were measured by ELISA (eBioscience)[21].

Intravital imaging

Cxcr6$^{GFP/+}$ CD11c$^{YFP/+}$ mice were i.p. injected weekly with 2 µg α-GalCer or vehicle, treated during 10 days with 2.17-mAlb or control BcII10-mAlb nanobody (i.p., 200 µg mouse^{-1} day^{-1}) and weighted daily as described above. On day 14, mice were anesthetized (ketamine/xylazin) and tail vein cannulation was performed to provide a route for i.v. compound administration. Mice were held in a custom-built stabilization device and further anesthetized with isoflurane. The liver was surgically exposed in the imaging chamber at 36°C and continuously superfused with 36 °C PBS. Imaging was performed on a LSM710 confocal microscope (Carl Zeiss) equipped with MaiTai Deep-See multiphoton laser (Spectra-Physics) and excitation wavelength at 890 nm. Baseline control iNKT cell speed was recorded 1 hour prior to i.v. injection with 2 µg α-GalCer. Imaging was continued 3 hours post-injection, after which i.v. injection with tetramethylrhodamine-labeled 70 kDa dextran (Life Technologies) was given as quality control. Images were analyzed with ImarisTrack (Bitplane) to quantify iNKT cell speeds (µm/min) over time. iNKT cell velocities after α-GalCer injection relative to average pre-injection speed for the same mouse were calculated and displayed as moving average of 10 frames for each time point.

Statistical analysis

Results are expressed as mean ± SEM. Sample sizes were estimated through power analyses using G*Power3.1 (http://www.gpower.hhu.de). Student t-tests, one-way ANOVA with Bonferroni correction and non-parametric Mann-Whitney U-tests were used to address statistical significance.

For intravital imaging Linear Mixed Models for Repeated Measures were used. P values <0.05 were considered statistically significant.

Results

Increased leptin levels and leptin receptor expression on iNKT cells in MM

To investigate a potential role for leptin signaling in MM, serum leptin levels were assessed in newly diagnosed MM (NDMM) patients next to age-matched patients diagnosed with monoclonal gammopathy of undetermined significance (MGUS) and healthy controls (HC). Significant increases of serum leptin levels were seen in NDMM patients relative to HC and MGUS patients (Figure 1a). Although a positive correlation was seen between body mass index (BMI) and serum leptin levels in HC and MGUS patients this correlation was totally absent in NDMM patients, suggesting that leptin levels in these patients were influenced by disease status rather than being related to physical features (Figure S1). Interestingly, significantly higher percentages of T cells from NDMM patients expressed the leptin receptor (LR) (Figure 1b). As previously reported, iNKT cells were reduced in NDMM patients (data not shown)[8–12]. Detectable blood and BM residing iNKT cells in NDMM patients expressed markedly higher levels of LR (Figure 1b). Of note, LR expression levels were even higher for BM derived cells as compared to cells in circulation underscoring the relevance for MM disease (Figure 1b). Moreover, the LR increase was clearly higher within the iNKT compartment compared to mainstream T cells (Figure 1b).Similar results regarding serum leptin levels and LR expression were obtained in the murine 5T33MM model, a well-validated pre-clinical immunocompetent model mimicking human MM disease closely(Figure 1c and 1d)[14,22].

MM cells and leptin synergistically counteract iNKT anti-tumor effects

In order to assess whether leptin has a direct or indirect impact on iNKT cell function in MM, co-culture experiments with NKT and MM cells (murine and human) were performed in absence or presence of exogenous leptin and/or LR blocking (Figure 2 and S2). Both in murine and human assays, high IFN-γ levels were observed when iNKT cells were stimulated with α-GalCer loaded dendritic cells (DC α-GalCer). However, IFN-γ levels dropped when DC α-GalCer stimulated mouse or human iNKT cells were co-cultured with respectively murine 5T33MMvv cells or human JJN-3 MM cells (Figure 2). When leptin was added to α-GalCer stimulated iNKT cells, decreases in IFN-γ levels were observed both for murine and human iNKT cells (Figure 2). Remarkably, iNKT cells co-cultured with MM cells (5T33MMvv or JJN-3 cells) and leptin completely lost their capacity to secrete IFN-γ (Figure 2). These results were confirmed in co-culture assays using other murine (5T33MMvt, 5T2MM) and human (RPMI 8226, U-266) MM cell lines (Figure S2). To examine whether this also

impacts conventional T cells, human co-culture experiments were set up with iNKT cell depleted T cells in presence of aCD3/CD28 Abs. Of interest, addition of MM cells combined with leptin caused much less profound reductions in T cell derived IFN-γ levels (data not shown), suggesting iNKT cells seem to be particularly prone to the dual effect of MM and leptin mediated inhibition of cytokine production. As expected LR blockade neutralized effects of exogenous added leptin on iNKT cell IFN-γ production (Figure 2). Altogether these data suggest that a combination of MM cells and leptin synergistically counteracts the iNKT cell anti-tumor function.

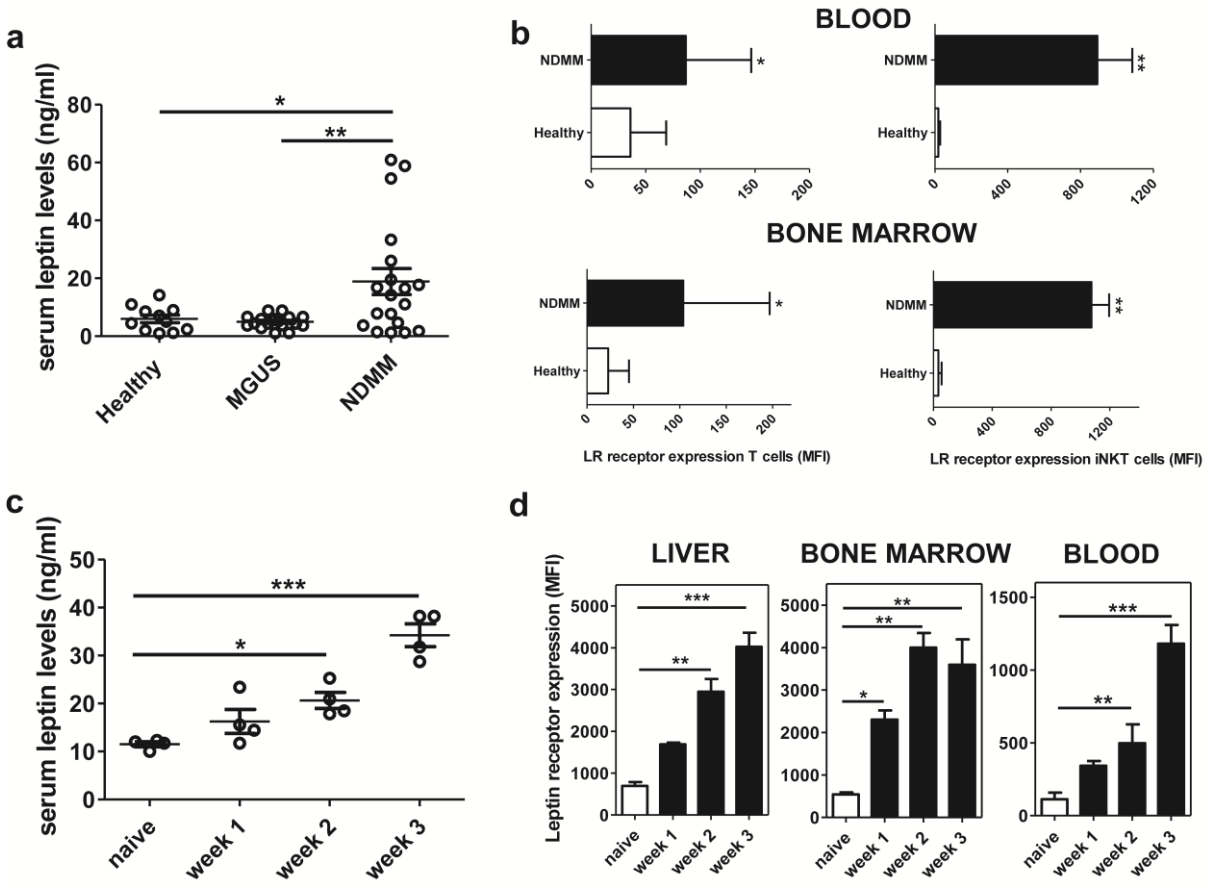

Figure 1: Leptin and its receptor on iNKT cells in MM. A) Serum leptin levels of NDMM patients (n=19) are compared with MGUS patients (n= 18) and healthy controls (n= 11). B) Mean Fluorescence Intensity (MFI) of leptin receptor expression on T and iNKT cells in blood and BM of NDMM compared to healthy individuals. For iNKT cell data analysis, a minimum cutoff of 500 events was set to allow proper statistical analyses. C) Leptin serum levels measured in 5T33MM WT mice at week 1, 2 and 3 after inoculation (n=4) compared to naive C57BL/KaLwRij mice (n=4). D) MFI of leptin receptor expression on iNKT cells in liver, bone marrow, spleen and blood at week 1, 2 and 3 after inoculation with 5T33MM cells compared to leptin receptor levels in naive C57BL/KaLwRij mice (n=3). *Natural killer T cells (NKT); Leptin (L); Leptin receptor (LR); Monoclonal gammopathy of undetermined significance (MGUS); Newly diagnosed multiple myeloma (NDMM). * p<0.05, ** p<0.01, *** p<0.001*

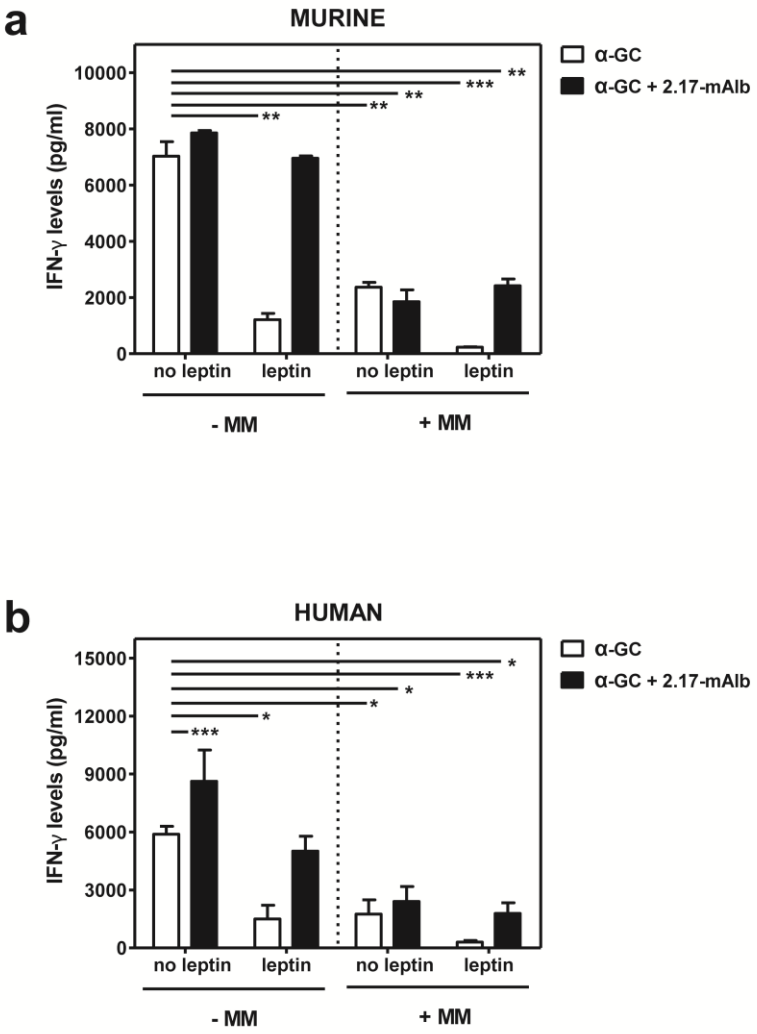

Figure 2: iNKT co-cultures with leptin and 2.17-mAlb Leptin receptor antagonist. A) Mean IFN-γ levels of murine iNKT co-culture experiments. The experiment was performed with 5T33vv MM cells. Respective conditions are iNKT cells + α-GalCer-loaded DC's; iNKT cells + α-GC-loaded DC's + MM cells; iNKT cells + α-GC-loaded DC's + MM cells + 2.17-mAlb; iNKT cells + α-GC-loaded DC's + 2.17-mAlb; iNKT cells + α-GC-loaded DC's + leptin; iNKT cells + α-GC-loaded DC's + leptin + MM; iNKT cells + α-GC-loaded DC's + leptin + 2.17-mAlb. B) IFN-γ levels of human iNKT co-culture experiment with JJN-3 human MM cell line. Human co-culture experiments were performed using the same set-up as murine co-culture experiments. *Natural killer T cells (NKT); Vivo (vv); Vitro (vt); Dendritic cells (DC); Alpha-Galactosylceramide (α-GC); Leptin Receptor antagonist (2.17-mAlb); Leptin (L). * p<0.05, ** p<0.01, *** p<0.001*

Leptin receptor blockade boosts iNKT mediated MM tumor protection

To investigate functional impacts of leptin signalling in iNKT cells towards MM tumor progression in the 5T33MM model (Figure 3a), we tested treatment with α-GalCer combined or not with a specific LR blocking nanobody (2.17-mAlb)[18]. To account for direct effects of iNKT cells, similar experiments were performed in mice backcrossed to a CD1d$^{-/-}$background, thereby lacking CD1d restricted NKT cells. As expected from leptin metabolic features, weight gain was observed in mice receiving LR antagonists, while this remained stable in control treated mice (Figure 3b). Consistently, fat pad weights significantly increased upon treatment with 2.17-mAlb (Figure 3c) while spleen and liver weights remained unchanged (data not shown). As expected, serum IFN-γ levels significantly increased in 5T33MM WT mice, in both control and treated groups receiving primary α-GalCer injections (day 1, Figure 4a), while in CD1d$^{-/-}$mice no cytokine release upon α-GalCer stimulation was observed (Figure S3a). Restimulation with iNKT cell agonists such as α-GalCer results in blunted cytokine responses caused by iNKT cell anergy, a well-described hyporesponsive state[23–25]. Consistent herewith, α-GalCer restimulation in 5T33MM WT mice did not lead to significant IFN-γ cytokine release upon secondary stimulation (Figure 4a). Surprisingly however, 5T33MM WT mice receiving the combination with LR antagonist displayed substantial serum IFN-γ levels after second α-GalCer restimulation (Figure 4a) while this was absent in 5T33MM CD1d$^{-/-}$mice (Figure S3a). We also evaluated the impact of LR blockade on α-GalCer restimulation in non-diseased (naive) C57BL/KaLwRijHsd mice. Here, partial rescue of iNKT cell functionality (IFN-γ secretion) could also be observed (Figure 5a), and comparable findings were measured in conventional C57BL/6 mice (data not shown).

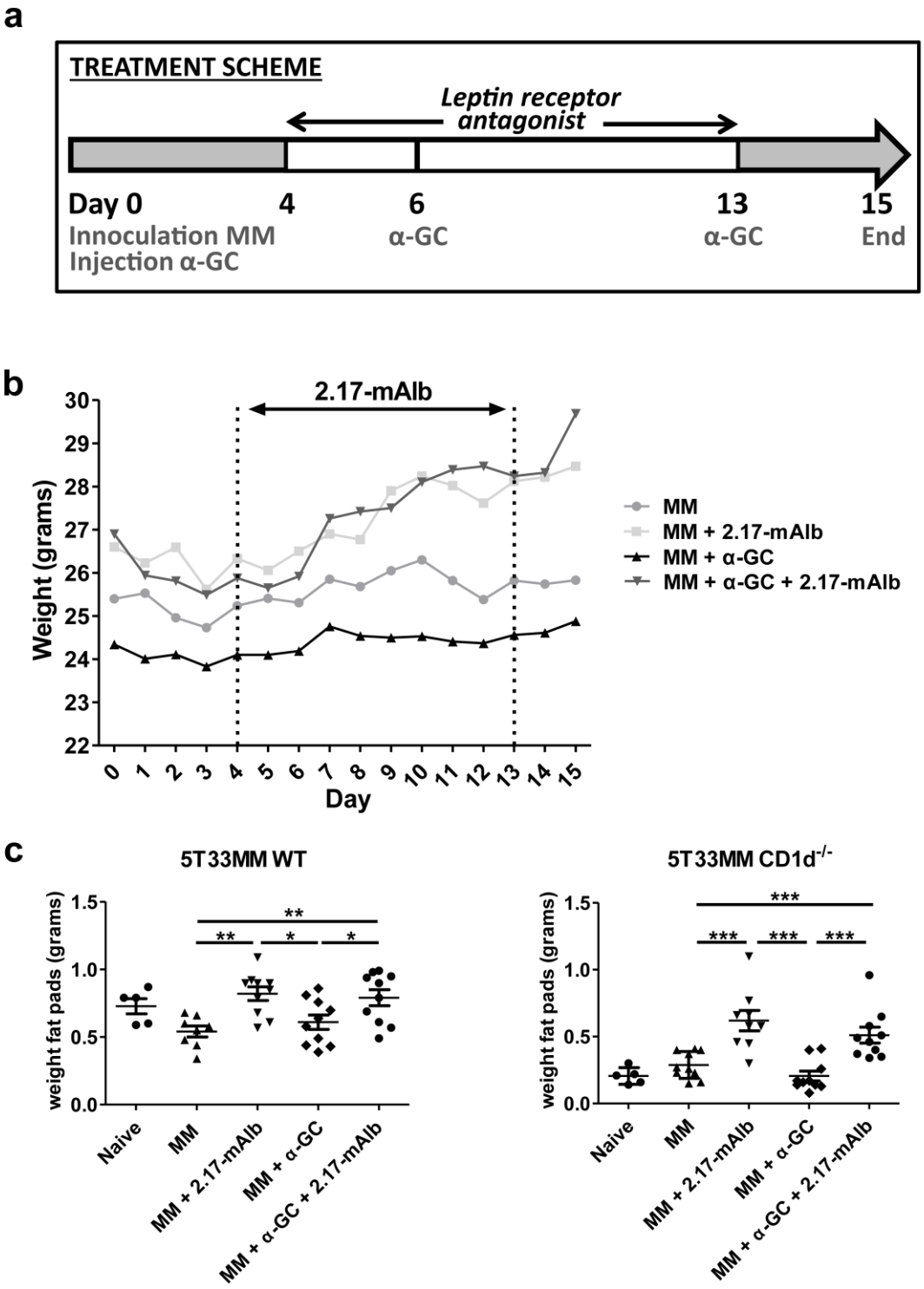

Figure 3: *In vivo* treatment with 2.17-mAlb Leptin receptor antagonist and α-GalCer. A) Schematic overview of the experiment. 5T33MM WT and 5T33MM CD1d$^{-/-}$ mice were inoculated together with 2µg α-GalCer at day 0. α-GalCer was re-injected weekly at day 6 and day 13. Injections with leptin antagonist 2.17-mAlb (200µg/mice) were started at day 4 and repeated during 10 days. Treatment groups were naive (n=5), MM (n=8), MM + 2.17-mAlb (n=10), MM + α-GalCer (n=10), MM + α-GalCer + 2.17-mAlb (n=10). B) Mice were weighted daily. Weight curves from one 5T33MM WT mouse representative for each group are shown. Similar weight curves were seen for 5T33MM CD1d$^{-/-}$ mice (data not shown). C) Fat pad weight at the end of the experiment for both 5T33MM WT and 5T33MM CD1d$^{-/-}$ mice. *Alpha-Galactosylceramide (α-GC); Leptin Receptor antagonist (2.17-mAlb); WT (wild type).* p<0.05, ** p<0.01, *** p<0.001*

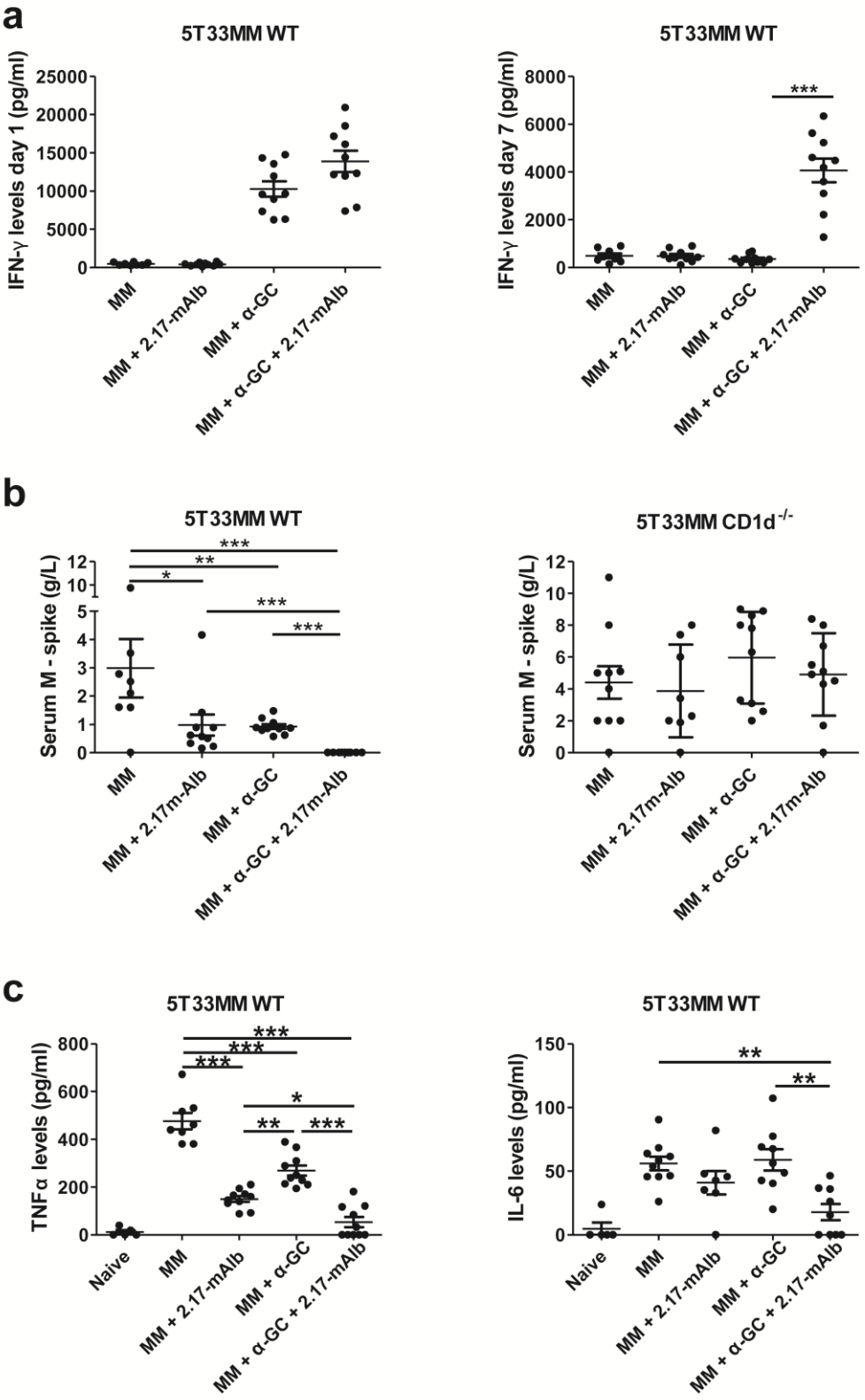

Figure 4: Combination effects of leptin receptor blockade and iNKT cell activation in MM *in vivo*. A) Serum IFN-γ levels 16h after first stimulation and second stimulation with α-GalCer are for 5T33MM WT mice. B) Serum M-spike in 5T33MM WT and CD1d$^{-/-}$mice. C) Serum TNFα and IL-6 levels at the end of the experiment for each treatment group in 5T33MM WT mice. *Alpha-Galactosylceramide (α-GC); Leptin Receptor antagonist (2.17-mAlb); Tumor necrosis factor alpha (TNFα); Interferon gamma (IFN-γ); Interleukine 6 (IL-6); Wild type (WT). * p<0.05, ** p<0.01, *** p<0.001*

A striking feature of iNKT cells is their motility pattern in organs where they reside such as liver. Because ligand induced iNKT cell activation is associated with motility arrest, we hypothesized that observed effects on altered cytokine release by iNKT cells in presence of LR neutralization may be reflected in altered iNKT cell dynamics. Therefore we utilized intravital dual photon microscopy in $Cxcr6^{GFP/+}$ $CD11c^{YFP/+}$ reporter mice[26,27] in steady state condition and upon a-GalCer stimulation. Under steady state conditions, iNKT cells rapidly migrate through liver sinusoids to scan their environment for potential triggers (Supplementary Video 1 and 2)♦. After primary injection with α-GalCer, iNKT cells rapidly go into antigen-induced motility arrest as part of their activation (Supplementary Video 1 and 2)♦ leading to steep reductions in measured iNKT cell speed (Figure 5b, red line). Similar arrests upon primary stimulation were also seen with LR antagonism (Figure 5b, purple line). In striking contrast, restimulation with α-GalCer failed to induce a ligand-induced iNKT cell arrest, which mirrors decreased cytokine production responses in anergic iNKT cells (Figure 5b, blue line and supplementary video 3)♦. Interestingly, α-GalCer restimulation in presence of LR blockade showed partial rescue of antigen-driven iNKT cell deceleration (Figure 5b green line and Supplementary Video 4)♦. Taken together, our data indicate that leptin signaling significantly impacts iNKT cell responsiveness upon repeated stimulation with α-GalCer by both influencing iNKT cell surveillance and cytokine production. This effect seems even more pronounced in a MM setting where leptin levels are progressively increased.

♦ Link to videos: https://drive.google.com/drive/folders/0B3BC3mG2v5kybmEzcDFWX2JrV0U?usp=sharing

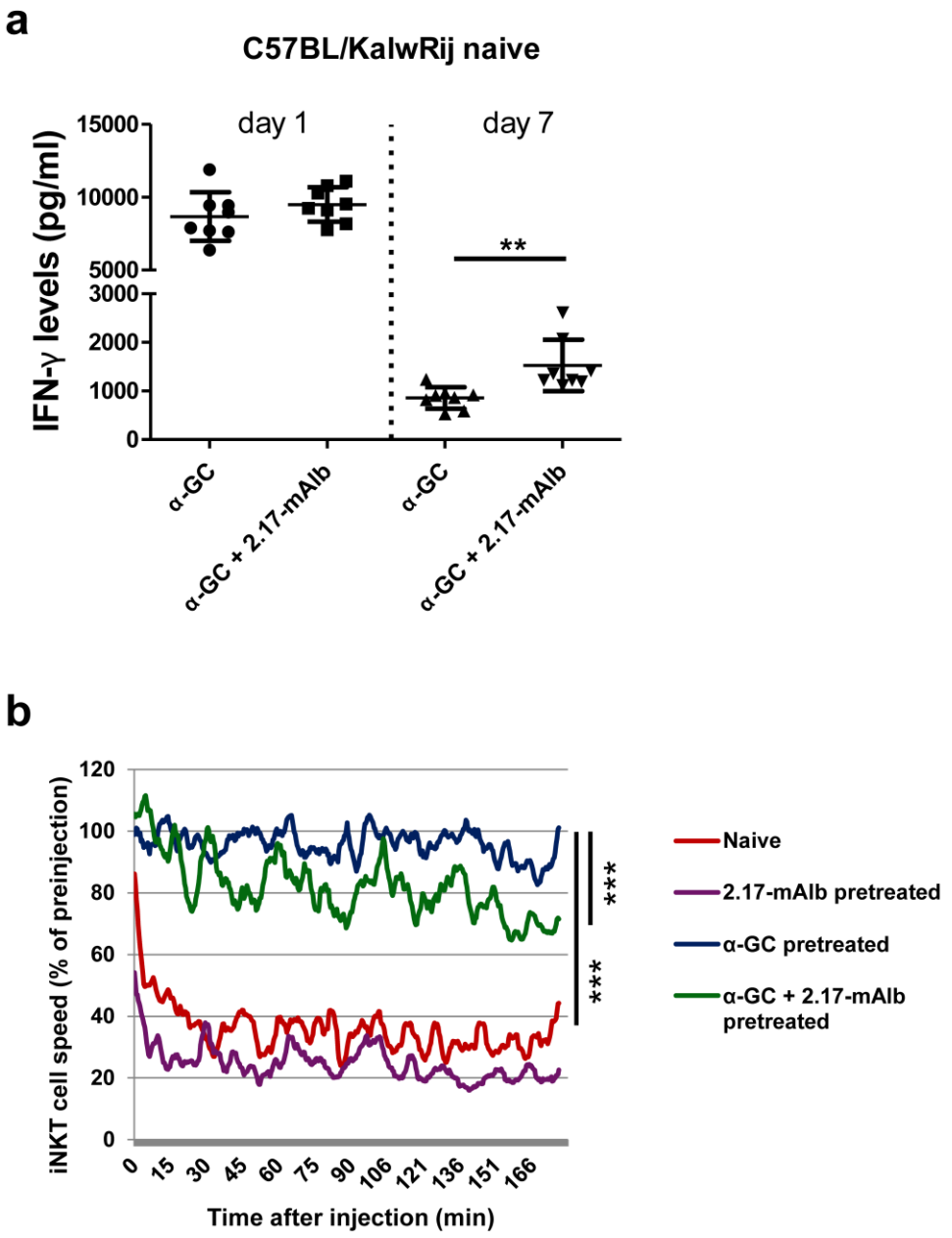

Figure 5: Effects of leptin receptor blockade and α-GalCer stimulation in MM-free mice. A) Serum IFN-γ levels of naive C57BL/KalwRij mice after first stimulation and second stimulation with α-GalCer, with and without leptin receptor antagonist co-treatment. B) Results show a ratio of the iNKT cell speed (µm/min) after injection with α-GalCer, relative to the preinjection speed (µm/min) within the same mouse. *Alpha-Galactosylceramide (α-GC); Leptin Receptor antagonist (2.17-mAlb); Interferon gamma (IFN-γ). ** p<0.01, *** p<0.001*

Based on these results we hypothesized that LR signaling blockade combined with iNKT cell stimulation could lead to even more efficient and synergistic anti-MM effects *in vivo*. In agreement with previous studies, α-GalCer stimulated iNKT cells significantly decreased BM tumor load and serum M-spike levels in 5T33MM WT mice (Figure 4b)[10,28]. However, therapeutic responses were suboptimal as a substantial fraction of mice did not respond sufficiently. Marked synergy was observed when repeated α-GalCer injections were combined with 2.17-mAlb treatment. Only very low measurable serum M-spike levels opposed to single (α-GalCer or 2.17-mAlb) treatments in 5T33MM WT mice were observed (Figure 4b). Anti-tumor effects were reflected by marked changes in serum TNFα and IL-6 concentrations (Figure 4c). Remarkably, mice treated with LR antagonist alone also showed significantly reduced M-spike levels (Figure 4b), even though no increased IFN-γ levels were observed during treatment (Figure 4a). However, within 5T33MM CD1d$^{-/-}$mice no differences in disease and cytokine profile were detected between treated mice indicating that LR blocking effects using glycolipid antigens were strictly iNKT cell dependent (Figure 4b and S3b).

iNKT cell stimulation combined with LR antagonism induces enhanced activation, proliferation and cytokine release of iNKT cells in MM

To further investigate how iNKT function is modulated by leptin in MM we restimulated iNKT cells with α-GalCer with or without 2 days of LR antagonist treatment and assessed their activation, proliferation and cytokine profile. α-GalCer stimulation led to iNKT cell upregulation of CD69 and CD25 levels in liver, spleen, BM and blood and also resulted in bystander activation of NK cells (Figure 6a and S4a). Combination of α-GalCer and 2.17m-Alb, however markedly augmented iNKT and NK activation. These observations were absent in control MM mice, mice only treated with 2.17mAlb nanobody and in 5T33MM CD1d$^{-/-}$mice (Figure 6a and S4a). Additionally, the α-GalCer and LR blockade combination also displayed higher fractions of BrdU positive iNKT and NK cells versus other treatment groups (Figure 6b and S4b). As expected, this effect remained absent in 5T33MM CD1d$^{-/-}$ mice (data not shown). We also determined the impact of combined α-GalCer -2.17mAlb treatment on iNKT IFN-γ and TNFα cytokine secretion in response to restimulation with α-GalCer. In line with altered dynamics and more persistent cytokine release, intracellular iNKT IFN-γ and TNFα levels were preserved in presence of LR antagonism, in contrast to controls (Figure 6c). Effects were also visible on NK cells, although the levels were much less pronounced (data not shown).

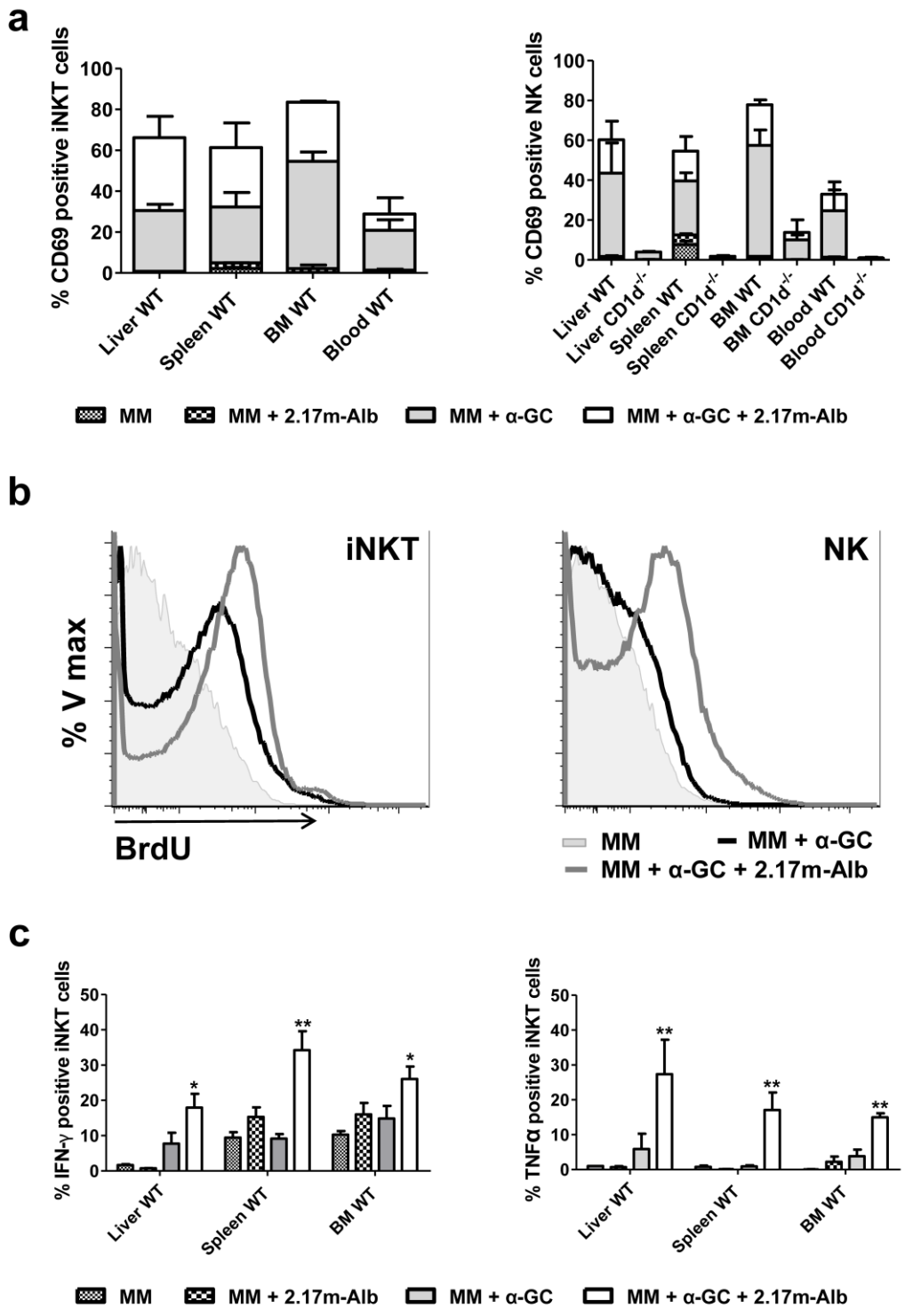

Figure 6: Activation, proliferation and cytokine secretion of NKT and NK cells. A) Percentage expression levels of activation marker CD69 for iNKT and NK cells in liver, spleen, bone marrow and blood. B) Fluorescence histograms representing BrdU incorporation of iNKT and NK in the liver of 5T33MM WT mice. C) Percentage of TNFα and IFN-γ positive iNKT and NK in liver, spleen and bone marrow of 5T33MM WT mice. *Alpha-Galactosylceramide (α-GC); Leptin Receptor antagonist (2.17-mAlb); Tumor necrosis factor alpha (TNFα); Interferon gamma (IFN-γ); Wild type (WT). * p<0.05, ** p<0.01*

Discussion

Our data provide several new and unexpected results which underscore the instrumental role of the adipokine leptin in MM progression by modulating iNKT cell mediated anti-tumor immunity (For an overview of the findings see Figure 7).

We observed increased serum leptin levels and LR expression levels on predominantly iNKT cells in NDMM patients and in the immunocompetent 5T33MM mice, a well-validated preclinical model for MM. Importantly, leptin levels were independently from BMI values linked to NDMM, which point to a role for an aberrant LR signaling in MM pathology. It should be noted that serum leptin and LR expression levels showed a bimodal distribution in NDMM patients, suggesting that the potential leptin-LR dysregulation may not affect all NDMM patients. Alternatively, this could reflect differences in degree of BM fatty deposition, which remains to be determined. Interestingly, iNKT cells make up a large proportion of lymphocytes in white adipose tissue (WAT)[29,30]. Moreover, they seem to have a striking role in regulating WAT inflammation, metabolism and weight control and are poised towards anti-inflammatory cytokine production as they closely interact with adipocytes in a CD1d dependent manner[6–8,31]. Obesity in humans and murine models is associated with a greatly reduced iNKT cell population and decreased CD1d expression levels. In this regard, increase in adipocyte composition or leptin/LR expression in MM BM could directly influence iNKT cell features. Indeed, both murine and human iNKT-MM cell co-culture assays revealed both MM and leptin to independently downmodulate the iNKT pro-inflammatory (i.e. anti-tumor) function. These profound immunosuppressive effects of leptin linked with cancer and particularly on human iNKT cells is a compelling novel observation. Leptin directly suppresses iNKT cells by modulating cytokine production, expansion and activation. While MM cells in BM of MM patients express the LR, they do not secrete leptin themselves (data not shown), suggesting that leptin is only secreted by adipocytes in the BM microenvironment[3]. However, MM cells do secrete different immunomodulating factors, such as IL-6 and IL-16 which could potentially inhibit iNKT cell function *in vivo*[32,33]. Leptin also directly promotes MM proliferation which, by increasing tumor load, may undermine iNKT cell mediated anti-tumor immunity[3].

Blocking LR signalling on α-GalCer activated iNKT cells leads to superior tumor protection *in vivo*. Indeed, dual treatment in 5T33MM WT mice resulted in remarkable reduced serum M-spike levels versus only a partial effect when α-GalCer alone was given. In addition, strong reduction in TNFα and IL-6 levels in MM mice confirmed the major impact blocking leptin signaling has on MM. TNFα and IL-6 are important cytokines involved in MM pathogenesis and are known to be secreted by BM stromal

cells and MM cells which in turn directly and/or indirectly influence MM cells in autocrine and paracrine manners[34].

It cannot be excluded that leptin induces primary or bystander effects on other immune cells. However, our data are consistent with a primary role for iNKT cells as targets affected by leptin in context of MM. First, LR levels were most abundantly expressed on iNKT cells versus other T cells and other lymphocytes such as NK cells. Secondly, effects of LR blockade on MM development were lacking in 5T33MM CD1d$^{-/-}$mice. Both leptin and α-GalCer mediated tumor suppressive effects were absent, which underscore a major role for leptin in modulating iNKT cell driven protective responses in MM. LR blockade resulted in enhanced iNKT cell activation and consistently increased iNKT cell production of IFN-γ and TNFα, which in turn leads to downstream activation of NK cells. This was also found to be absent in CD1d$^{-/-}$mice treated with LR blockade. It should be noted that under steady state conditions, iNKT cell deficiency, did not lead to enhanced tumor growth in the 5T33MM model. However, given profound production of leptin during MM development, anti-tumor protective effects of iNKT cells become gradually overruled. In line with this, LR blockade had a striking impact on MM-development as measured by M-spike levels, an observation that was clearly undetectable in the absence of iNKT cells. This suggests that effects of leptin antagonism are iNKT cell dependent.

Unexpectedly, we found one of the major mechanisms underlying enhanced protective effects of *in vivo* iNKT cell activation in presence of LR antagonism be caused by its ability to reverse or prevent anergy. Thus, combining α-GalCer with LR blockade strongly amplified and prolonged iNKT cell activation and modulated iNKT threshold activation upon repeated α-GalCer administration. Anergy induction represents a major drawback in efficacy of iNKT based therapies [23,35]. By means of intravital microscopy, we are the first to provide a detailed time course of iNKT cell dynamics in response to α-GalCer. We observed that ligand-induced TCR-mediated iNKT cell arrest, one of the earliest events in iNKT cell stimulation, was blunted under conditions of restimulation. Thus, rather than inducing a TCR mediated arrest, iNKT cells retained motility in liver upon restimulation. Importantly, we demonstrated that LR antagonism partially rescued this iNKT cell motility arrest, suggesting that the exhausted antigen-mediated iNKT cell function can be partially restored by leptin blockade and further extends the notion that this may interfere with TCR-mediated signaling under certain conditions. Interestingly, the therapeutic impact, e.g. increase in IFN-γ release by restimulated iNKT cells upon LR blockade, was profoundly amplified in the MM setting where leptin levels are particularly elevated compared to healthy mice, presumably secondary to increased BM fatty infiltration and upregulation of LR on iNKT cells. While previous studies highlighted an intriguing relationship between adipose tissue, antigen presentation of glycolipid antigens and iNKT cells, our

data provide an alternative explanation of the relation between adipocytes and iNKT cells through release of adipokines.

Underlying mechanisms of leptin signaling blockade on iNKT cell anergy are not entirely clear. It can be anticipated that aberrant leptin levels and LR expression significantly impact TCR-mediated signaling directly by influencing iNKT cell activation threshold and function but also indirectly by an effect on the programmed cell death protein 1 (PD-1) pathway, clearly linked to iNKT cell anergy[23,35]. These mechanisms are not mutually exclusive and could have a synergistic effect. However, other mechanisms may also be involved in this context, LR stimulation results in JAK2 transphosphorylation and activation of several signaling cascades including STAT3, MAPK, PI3K and mTOR pathways [36–39]. Intriguingly, tuberous sclerosis 1 (TSC1), negative regulator of mTOR signaling, has been linked to iNKT cell anergy as TSC1 deficiency in iNKT cells results in resistance to α-GalCer-induced hyporesponsiveness and correlated with impaired upregulation of PD-1[40]. The mTOR pathway seems also involved in direct cell growth, proliferation and migration of MM cells and mTOR kinase inhibitors and dual inhibitors of PI3K/mTORC1 have showed promising *in vitro* and *in vivo* effects in preclinical MM studies[41]. JAK2 and STAT3 inhibitors have also led to downregulation of PD-L1 in non-small lung cancer cells which could be compatible with a role of leptin-induced JAK/STAT signaling in inducing anergy and immunosuppression in MM as described here[42]. In any case, our data support the concept that hyperleptinemia is promoting MM tumor progression and iNKT cell dysfunction and underscores the leptin-LR axis acts as an immune checkpoint in MM.

In summary, our study unveils a previously unknown link between leptin and iNKT cells in MM immunosuppression. The data enforce a novel paradigm in which adipokines released by accumulating adipocytes within MM play an instrumental and crucial role in suppressing anti-tumor immunity which can be reversed by LR antagonism. Overall, pharmacological targeting of leptin signaling acting as a new checkpoint inhibitor represents a novel strategy for immunotherapy in MM.

Overview findings

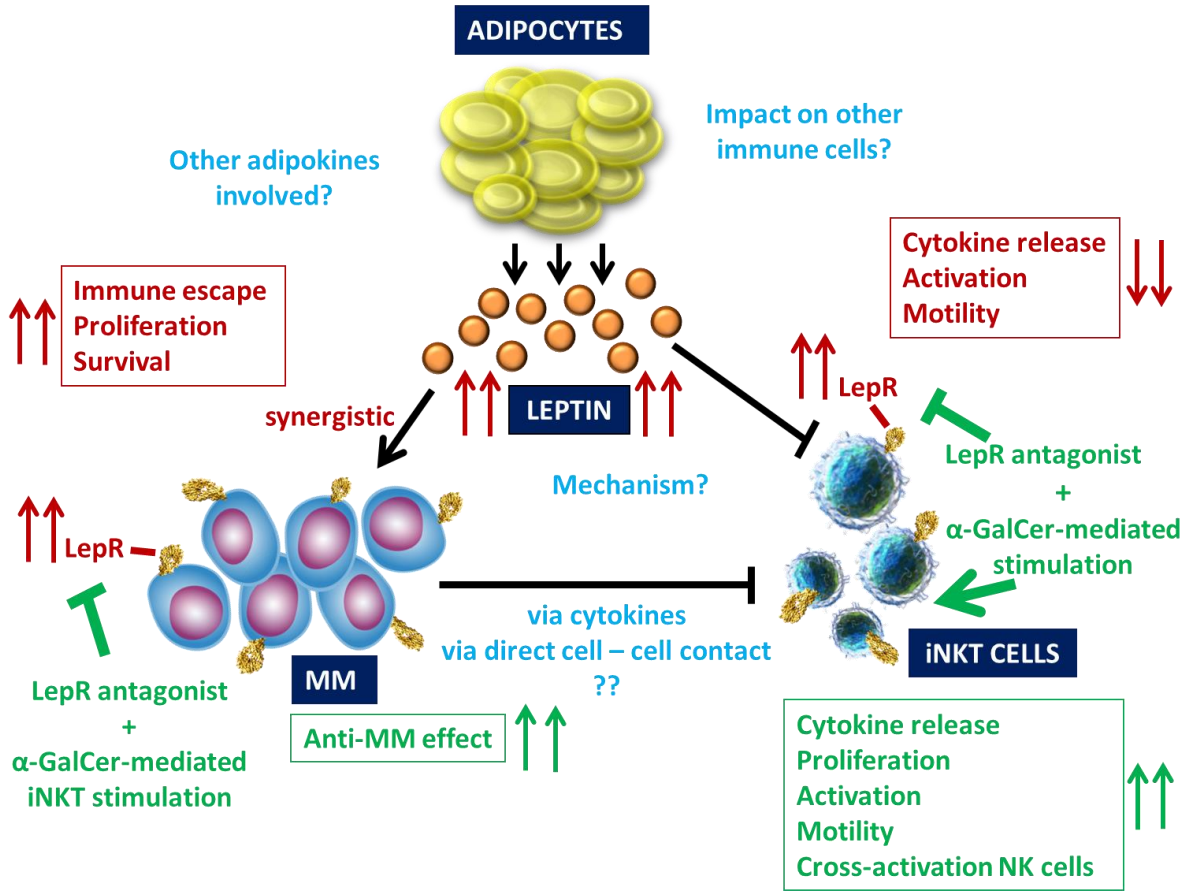

Figure 7: Overview general findings. We were the first to reveal the leptin – leptin receptor (LepR) axis as an important iNKT cell-mediating pathway in MM and anti-tumor immunity. Progressive increases of leptin secreted by adipocytes and LepR expression levels were observed on iNKT cells during MM progression (red upward arrows). Leptin proved to profoundly directly inhibit (black blocking arrow) the iNKT cell functionality and to stimulate MM cells (normal black arrow), reinforcing the inhibition on iNKT cells even more (synergistic effect leptin and MM cells on iNKT cells, black blocking arrow). iNKT cell stimulation combined (normal green arrow) with LepR antagonism (green blocking arrow) induced enhanced activation (alleviated anergy), proliferation, motility and cytokine release of iNKT cells in MM (green box with upward arrows). Moreover, cross-activation of NK cells could be observed (green box with upward arrows). An almost complete tumor protection was observed when the combination of α-GalCer-mediated iNKT stimulation and a LepR antagonist was used (green bow with upward arrows). However, several questions still remain unanswered (light blue) and will be the objective of future research. What are the mechanisms inducing a leptin increase in MM and what is the impact of leptin on other immune cells in MM? Are there also other adipokines involved? And by which leptin-stimulated mechanisms do MM cells contribute in iNKT cell impairment. *Alpha-Galactosylceramide (α-GalCer); Leptin Receptor (LepR); Leptin Receptor antagonist 2.17-mAlb (LepR antagonist); Multiple myeloma (MM).*

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Supplementary material and methods

Mice

Male C57BL/KaLwRijHsd mice were purchased from Envigo (Horst, The Netherlands) and used when they were 6 to 8 weeks old. Mice were housed and maintained following the conditions approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussels (license no. LA1230281). The animal ethics meet the standards required by the United Kingdom Coordinating Committee on Cancer Research Guidelines (UKCCCR, 1998). Ethical committee obtained by the VUB animal ethics committee with approval number 14_281_12. The 5T33MM and 5T2MM model originated spontaneously in C57BL/KaLwRij mice as previously described and have since been propagated *in vivo* by intravenous transfer of the diseased BM into young syngeneic mice, which in turn develop myeloma in 3-4 week and 10-12 weeks respectively[1,2].

C57BL/6 CD1d$^{-/-}$mice (The Jackson Laboratory) were backcrossed onto the C57BL/KaLwRijHsd background. The backcrossing was accelerated by the use of a marker-assisted accelerated backcrossing system (MAX BAX system, Charles River Laboratories). After 5 generations, C57BL/KaLwRijHsd CD1d$^{+/-}$mice were crossed to generate the final C57BL/KaLwRijHsd CD1d$^{-/-}$mice.

For the intravital imaging, C57BL/6 Cxcr6$^{GFP/GFP}$ and CD11c$^{YFP/YFP}$ mice (The Jackson Laboratory) were crossed to obtain the Cxcr6$^{GFP/+}$ CD11c$^{YFP/+}$ mice.

Cell lines

The 5T33MMvt cell line resulted spontaneously from cultured 5T33MMvv grown *in vitro,* independently from the BM stroma. RPMI 8226, JJN-3 (American Type Culture Collection) and U266 (ATCC) are three well characterized human cell lines that were selected for our experiments. They were cultured as previously described and regularly tested for mycoplasma contamination[1,3–5].

Generation of Dendritic cells and loading with α-GalCer

Murine BM progenitor cells were isolated by flushing the content out of the femur and tibia of naive mice with sterile PBS. DCs were cultured from these BM progenitor cells and plated in 100×20 mm cell culture dishes (Greiner Bio-One, GmbH, Germany) at 2×10^6/mL in RPMI 1640 GlutaMAX^{TM} medium (GIBCO, Life Technologies, Ghent, Belgium) supplemented with 10% FetalClone I (FCI, Hyclone, Logan, USA) and 1% Penicilin/Streptomycin (Lonza, Basel, Switzerland). 20 ng/mL of recombinant mouse granulocyte macrophage colony stimulating factor (recombinant murine GM-CSF, PeproTech, London, UK) and 50µM β-mercaptoethanol (β-ME, Sigma-Aldrich) were added to the culture at day 0. On day 3, 50% of the media was completely refreshed with fresh medium containing GM-CSF and β-ME. On day 6 media was refreshed again. On day 8, the immature DCs were harvested

and 10 ng/mL GM-CSF was used to mature DCs overnight together with 10 µg/mL α-GalCer or vehicle (0.5% Tween-20 in PBS, as a control). α-GalCer, dissolved in DMSO at 1 mg/mL was kindly provided by Prof. Dr. S Van Calenbergh (Laboratory of Medicinal Chemistry, University of Ghent, Belgium) and kept at −20°C. On day 9, after extensive washing, α-GalCer loaded/unloaded DCs are ready to be used for co-culture experiments.

Isolation and expansion iNKT cells

Six livers from C57BL/KaLwRij mice were removed, dissected, strained and pooled. The resulting cellular suspension was layered over a Percoll density gradient (GE Healthcare, Diegem, Belgium). Following centrifugation, liver mononuclear cells (MNCs) were collected and subsequently stained with APC-labelled CD1d/a-GalCer tetramer in combination with CD3 (17A2, eFluor©450, eBioscience, Vienna, Austria). Afterwards, they were purified by fluorescence activated cell sorting (FACS Aria II, BD Biosciences). FACS sorted iNKT cells were then resuspended in complete RPMI 1640 medium and cultured *in vitro* using a combination of recombinant murine IL-2 (10 ng/mL, eBioscience) and Il-12 (1 ng/ ml, eBioscience) together with plate-bound T cell receptor (TCR) (anti-CD3 and anti-CD28, eBioscience) before being expanded in the presence of murine recombinant IL-7 (eBioscience). Using this expansion technique, approximately 10^8 iNKT cells can be generated within 18-20 days of culture, after which they can be used for co-culture assays.

Isolation 5T33MMvv cells

For 5T33MM vivo (vv, primary MM cells) cells mice were sacrificed when terminally ill. The BM was flushed out of femurs and tibiae. The 5T33MM cells were subjected to red blood cell lysis. Viability was more than 95% and the purity was reaching more than 85%, as measured by flow cytometric analysis.

Flow Cytometry and antibodies

Liver, spleen were isolated as previously described[1,6] . BM cells were isolated as described earlier in the article. Murine lymphocytes were resuspended in staining buffer containing saturating amounts of anti-Fcγ Receptor type II/type III monoclonal antibodies (Miltenyi Biotec, Sunnyvale, CA). PE-labelled CD1d/a-GalCer tetramer was added at room temperature, 15 min before the addition of the mouse leptin R biotinylated antibody (R&D Systems, Minneapolis, MN), TCRβ (H57-597, APC-Cyanine7, eBioscience), CD3 (145-2C11, PE-Cyanine7, eBioscience), CD4 (RMA4-5, V500, BD HorizonTM) and NK1.1 (PK136, eFluor©450, eBioscience) during 30 min on ice. 7-AAD (BD PharmigenTM) was also included for the exclusion of death cells. After washing the cells, an anti-APC HRP labeled secondary antibody (eBioscience) was added against the mouse leptin R biotinylated

antibody. Living single cells were acquired on a FACSCantoII (BD Biosciences) flow cytometer and analyzed using FlowJo (TriStar) software. iNKT cells were identified as: $7AAD^{-}CD3^{+}$CD1d/a-GalCer $tetramer^{+}$ cells and subsequently further characterized for TCRβ, CD4, NK1.1 or leptin receptor expression. Immunostaining for BrdU was performed using the BrdU flow kit as per the supplier's instructions (Becton Dickinson). Mice were injected with BrdU in 0.1 ml of sterile PBS (100 mg/kg body weight) i.p. 4 h before sacrifice. For cytokine analysis intracellular stainings FITC-conjugated anti-IFN-γ (XMG1.2) and APC-conjugated anti-TNFα (MP6-XT22) antibodies (eBioscience) were used.

Human mononuclear cells were stained with 7-AAD (BD PharmigenTM) for the exclusion of death cells together with PE-conjugated anti-human Vα24Jα18 TCR (6B11) and Amcyan-conjugated anti-CD3ε (UCHT1) to identify the iNKT cells within the T cell population, subsequently Pacific Blue-conjugated anti-CD4 (OKT4), Alexa Fluor 700-conjugated anti-CD8 (OKT8) (all eBioscience) were used to distinguish the subpopulations, the APC-conjugated anti-human leptin receptor (R&D Systems) was used to determine leptin receptor expression on human iNKT cells. The staining were acquired on a FACSLSRII (Becton Dickinson). Analyses were performed using FlowJo software (Tree Star Inc.).

Supplementary references

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Supplementary figures

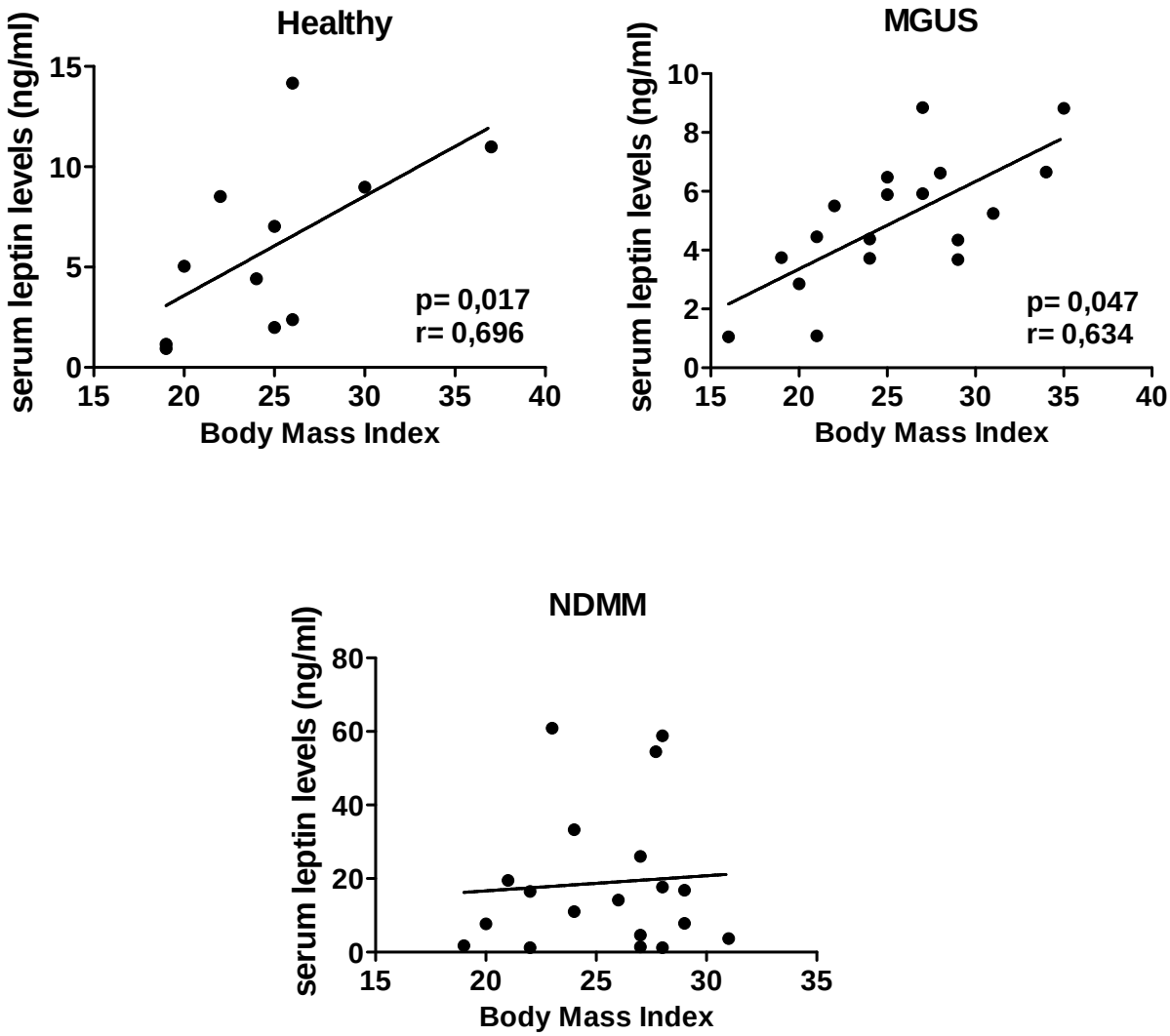


Figure S1: Correlation analysis between serum leptin levels (ng/ml) and body mass index in healthy control, MGUS and NDMM patients.

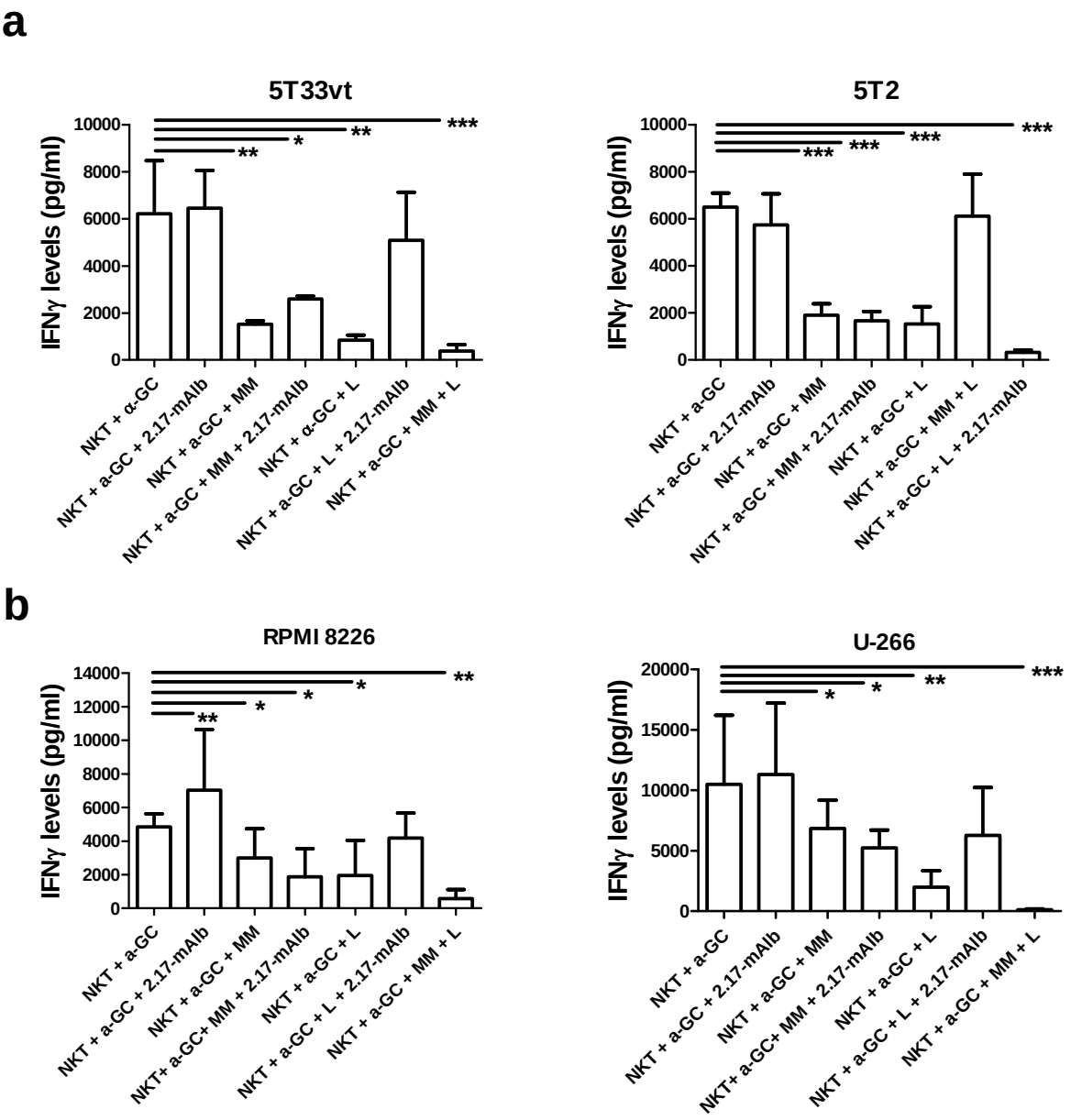


Figure S2: iNKT co-culture experiments with leptin and the 2.17-mAlb Leptin receptor antagonist. *A) Mean IFN-γ levels of murine iNKT co-culture experiments are illustrated. Experiments were performed with different murine MM cell lines: 5T33MMvt and 5T2MM. Respective conditions are iNKT cells + α-GalCer-loaded DC's; iNKT cells + α-GC-loaded DC's + MM cells; iNKT cells + α-GC-loaded DC's + MM cells + 2.17-mAlb; iNKT cells + α-GC-loaded DC's + 2.17-mAlb; iNKT cells + α-GC-loaded DC's + leptin; iNKT cells + α-GC-loaded DC's + leptin + MM; iNKT cells + α-GC-loaded DC's + leptin + 2.17-mAlb. B) IFN-γ levels of human iNKT co-culture experiment are illustrated. Human co-culture experiments were performed using the same set-up as the murine co-culture experiments. Different human MM cell lines were tested including RPMI 8226 and U-266. Natural killer T cells (NKT); Vivo (vv); Vitro (vt); Dendritic cells (DC); Alpha-Galactosylceramide (α-GC); Multiple myeloma cells (MM); Leptin Receptor antagonist (2.17-mAlb); Leptin (L). * p<0.05, ** p<0.01, *** p<0.001*

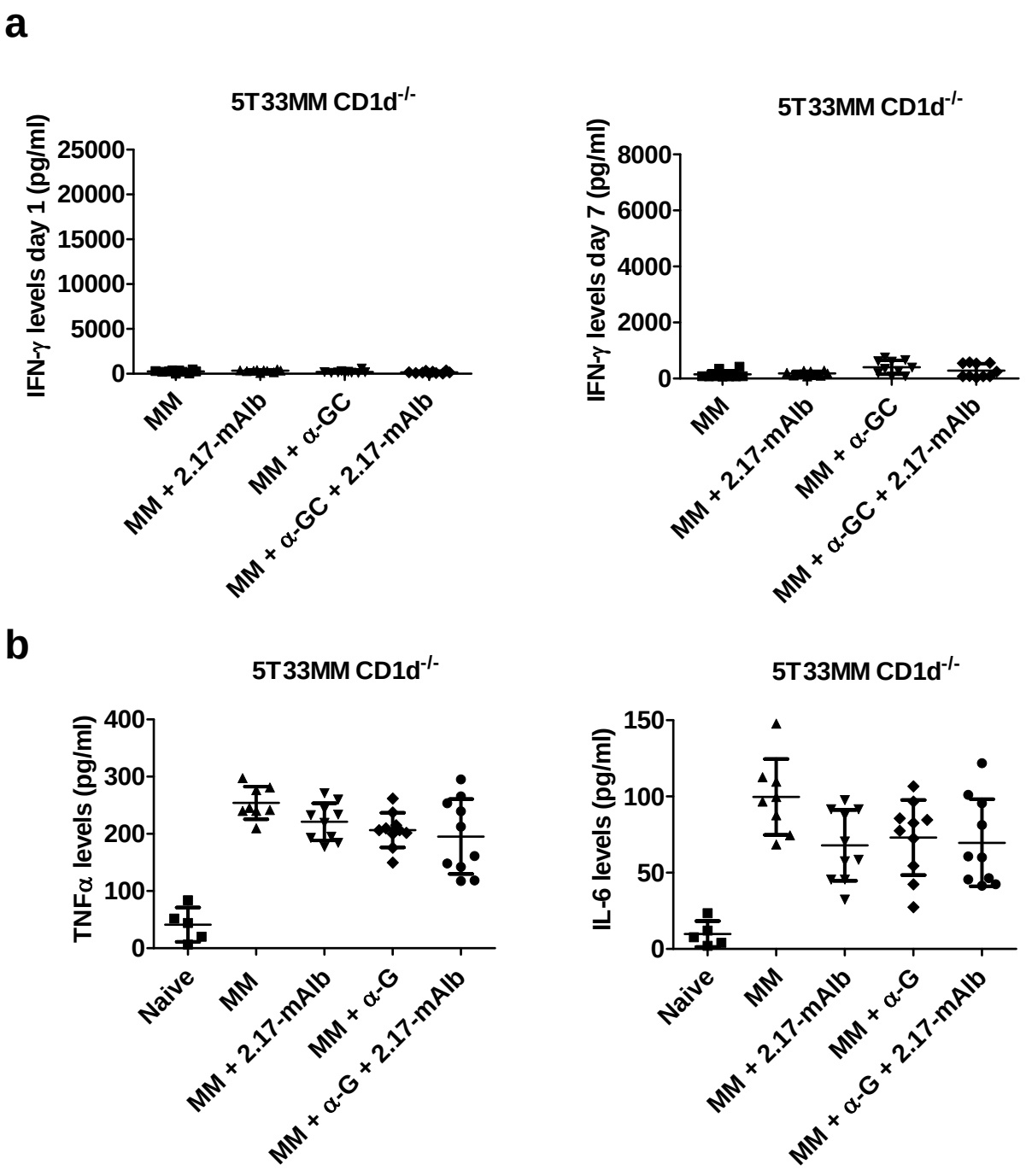
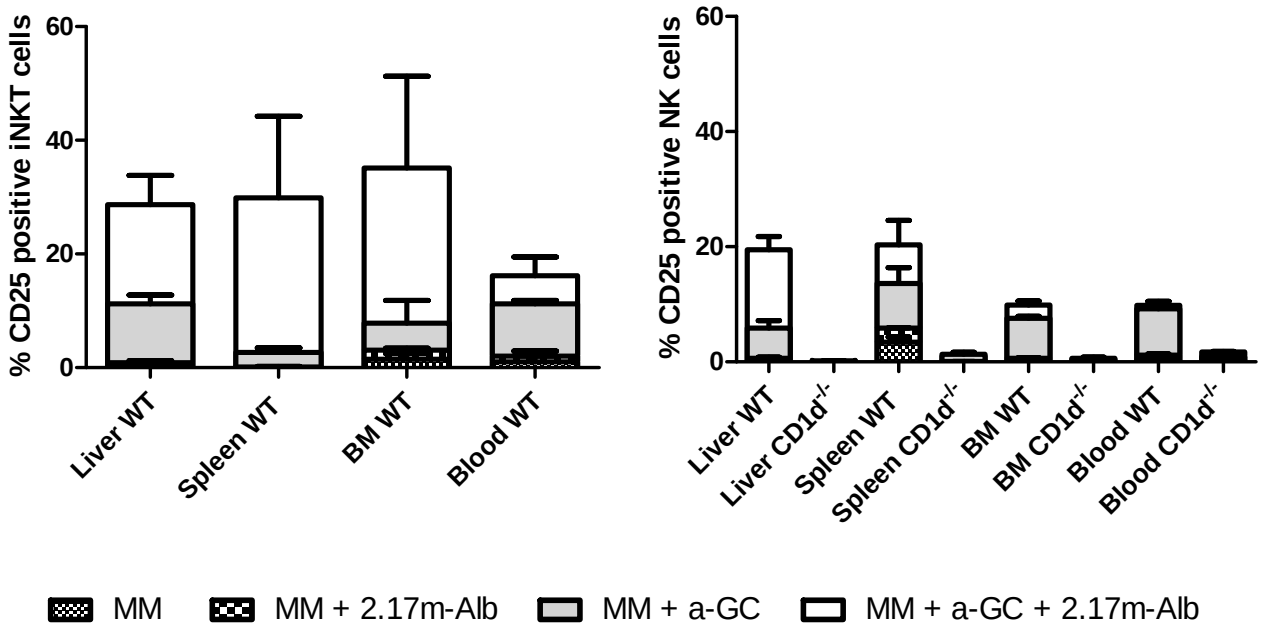


Figure S3: The combination effects of leptin receptor blockade and iNKT cell activation in 5T33MM C57BL/KaLwRij CD1d$^{-/-}$

*A) IFN-γ levels in the serum 16h after the first stimulation and second stimulation (one week later) with α-GalCer are represented. B) Serum TNFα and IL-6 levels were determined by ELISA at the end of the experiment for each group in 5T33MM C57BL/KaLwRij CD1d$^{-/-}$ mice. Alpha-Galactosylceramide (α-GC); Leptin Receptor antagonist (2.17-mAlb); Multiple myeloma (MM); Tumor necrosis factor alpha (TNFα); Interferon gamma (IFN-γ); Interleukine 6 (IL-6). * p<0.05, ** p<0.01, *** p<0.001*

a



b

% BrdU incorporation		iNKT cells	NK cells	
		5T33MM WT	*5T33MM WT*	*5T33MM CD1d$^{-/-}$*
	MM	0,6 ± 0,7	0,2 ± 0,1	0,1 ± 0,1
	MM + 2.17mAlb	0,4 ± 0,1	0,3 ± 0,1	0,1 ± 0,1
	MM +α-GalCer	15, 5 ± 6,8	2,5 ± 0,4	0,2 ± 0,1
	MM +2.17mAlb + α-GalCer	28, 3 ± 17, 0	7, 7 ± 4,1	0,1 ± 0,1

Figure S4: Evaluation of CD25 expression and proliferation of NKT and NK cells after leptin receptor blockade and iNKT cell activation. *A) Flow cytometry analysis was performed after second re-stimulation with 2µg α-GalCer and 2 days of initiated treatment with the leptin antagonist 2.17-mAlb (200µg/mice). 3 mice were evaluated in each treatment group in 5T33MM C57BL/KaLwRij and C57BL/KaLwRij CD1d$^{-/-}$ mice. Percentage expression levels of activation marker CD25 are illustrated for iNKT and NK cells in liver, spleen, bone marrow and blood of the different treatment groups. B) Table with an overview of the % BrdU incorporation ± SD of iNKT and NK cells in the liver for each treatment group in 5T33MM C57BL/KaLwRij WT and C57BL/KaLwRij CD1d$^{-/-}$ mice. Natural killer T cells (NKT); Natural killer cells (NK); Alpha-Galactosylceramide (α-GC); Multiple myeloma (MM) Leptin Receptor antagonist (2.17-mAlb)*

Supplementary table 1: Characteristics healthy controls, MGUS and NDMM patients

	Number	Mean	Range
Control individuals (n= 11)			
Age (years)		60	54-65
Gender (M/F)	5/6		
BMI[1]		24,8	19-37
Monoclonal gammopathy of undetermined significance (n=18)			
Age (years)		77	59-91
Gender (M/F)	12/6		
BMI[1]		25,4	16-35
Newly Diagnosed multiple myeloma (n= 19)			
Age (years)		69	55-83
Gender (M/F)	8/11		
BMI[1]		25,4	19-31
M-component type			
IgG	14		
IgA	3		
IgM	1		
IgD	1		
Light chain type			
Kappa	11		
Lambda	8		
ISS Stage[1]			
Stage I	7		
Stage II	7		
Stage III	5		
Adverse cytogenetics			
13q	5		
t(4,14)	2		
17p	3		

[1]**BMI: Body Mass Index; ISS: International Staging System**

Supplementary videos

Link to videos:

https://drive.google.com/drive/folders/0B3BC3mG2v5kybmEzcDFWX2JrV0U?usp=sharing

Supplementary Videos:

These videos show intravital time-lapse sequences of iNKT cells (green) in the liver prior to (left panel) and after injection with α-GalCer (right panel) in the same mouse. Blue signal corresponds to collagen fibers visualized by second harmonics. Red cells are CD11c-YFP positive (dendritic cells or majority of Kupffer cells).

Supplementary Video 1: α-GalCer-mediated iNKT cell arrest in the liver. A decrease in iNKT cell speed due to ligand-induced TCR-mediated arrest can be seen after injection with α-GalCer (right panel).

Supplementary Video 2: α-GalCer-mediated iNKT cell arrest after treatment with leptin receptor antagonist. Mice that were pretreated with vehicle and leptin receptor antagonist showed a decrease in iNKT cell speed due to ligand-induced TCR-mediated arrest after injection with α-GalCer (right panel).

Supplementary Video 3: Strongly blunted arrest response after α-GalCer injection in mice with anergic iNKT cells. In mice that were pretreated α-GalCer, the iNKT cells showed a dramatically reduced ligand-induced TCR-mediated arrest after renewed injection with α-GalCer (right panel).

Supplementary Video 4: Partial rescue of ligand-mediated arrest response after pretreatment with leptin receptor antagonist. Mice that were pretreated with α-GalCer and 2.17-mAlb showed a partial rescue of the ligand-induced TCR-mediated iNKT cell arrest after renewed injection with α-GalCer (right panel)

“The most exciting phrase to hear in science, the one that heralds new discoveries, is not “Eureka” but “That's funny...”

Isaac Asimov

Chapter V

"Both mucosal-associated invariant and natural killer T cell deficiency in multiple myeloma can be countered by PD-1 inhibition"

Letter to the editor published in Haematologica

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Both mucosal-associated invariant and natural killer T cell deficiency in multiple myeloma can be countered by PD-1 inhibition

PD-1 inhibition on invariant T cells in myeloma

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The authors declare no competing financial interest.

Keywords: Myeloma, iNKT cells, MAIT cells, Tumor immunity, PD-1

Mucosal-associated invariant T (MAIT) cells primarily contribute to immune defense against infectious pathogens and regulate pathogenesis of various inflammatory diseases[1]. They are innate-like T lymphocytes expressing an invariant TCR Vα7.2-Jα33 chain in humans and displaying high expression levels of CD161 and IL-18R. They are primarily localized in mucosal tissue and respond to vitamin B2 metabolites in an MHC class 1b (MR1)-dependent manner[1]. Despite a different ontogeny, MAIT cells share a close lineage relationship with iNKT cells, another invariant T cell subset recognizing glycolipids and important for anti-tumor immunity[1,2]. Moreover, it has been suggested that they share a common niche and could therefore be functionally redundant[3]. Recently, MAIT cells have been implicated in cancer and were shown to support anti-tumor immunity[4]; however their exact role remains a less explored area. Until now, most reports on involvement of MAIT cells in cancer has been limited to mucosa-associated cancers[5]. Recently however, Wallace et al reported MAIT cell deficiency in chronic lymphocyte leukemia (CLL), suggesting a possible involvement of MAIT cells in hematological malignancies as well[4]. Further investigation of MAIT cell functionality in cancer patients beyond mucosa-associated malignancy is therefore warranted. In multiple myeloma (MM), invariant T cells such as iNKT cells have been reported by us and others to be deficient[6]. Until now, this hasn't been investigated for MAIT cells and whether they prove to be important in MM is far from established. Their similarities with iNKT cells and regulatory functions, together with recent reports suggesting their possible involvement in non-mucosa associated malignancy, make them a logical candidate for further studies. Therefore, we examined the phenotype and functionality of MAIT cells in MM.

A total of 14 newly diagnosed MM (NDMM) patients and 14 age-matched healthy controls were enrolled in this study (clinical characteristics in supplementary Table 1). The frequency of MAIT cells ($CD3^+Vα7.2^+CD161^+$) in total $CD3^+$ lymphocytes was determined by flow cytometry in peripheral blood (PB) and bone marrow (BM) of NDMM patients and healthy counterparts (Figure 1A). MAIT cell percentages were significantly lower in NDMM patients compared to healthy individuals (Figure 1B). Reduced circulating MAIT cell numbers have also been reported in autoimmune disorders (such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis) and infectious diseases (tuberculosis, HIV) next to malignancies (CLL and mucosa-associated cancers) [4,5,7–11]; however we are the first to show MAIT cell disturbances in MM. MAIT cells can be subdivided into $CD4^+$, $CD8^+$ and double negative subsets (Figure 1C). In PB and BM, most of the MAIT cells carry either a $CD8^+$ or double negative phenotype. Analysis of MAIT cells in NDMM patients by flow cytometry revealed significantly lower percentages of the $CD8^+$ MAIT cell subset and the double negative fraction (Figure 1D), while the $CD4^+$ fraction remained unchanged (data not shown). Therefore, reduced $CD8^+$ and DN

subset levels seem to contribute to total MAIT cell number reduction in MM. Similar observations were published for SLE, HIV and multiple sclerosis but were not yet reported in other cancers[8,10,11].

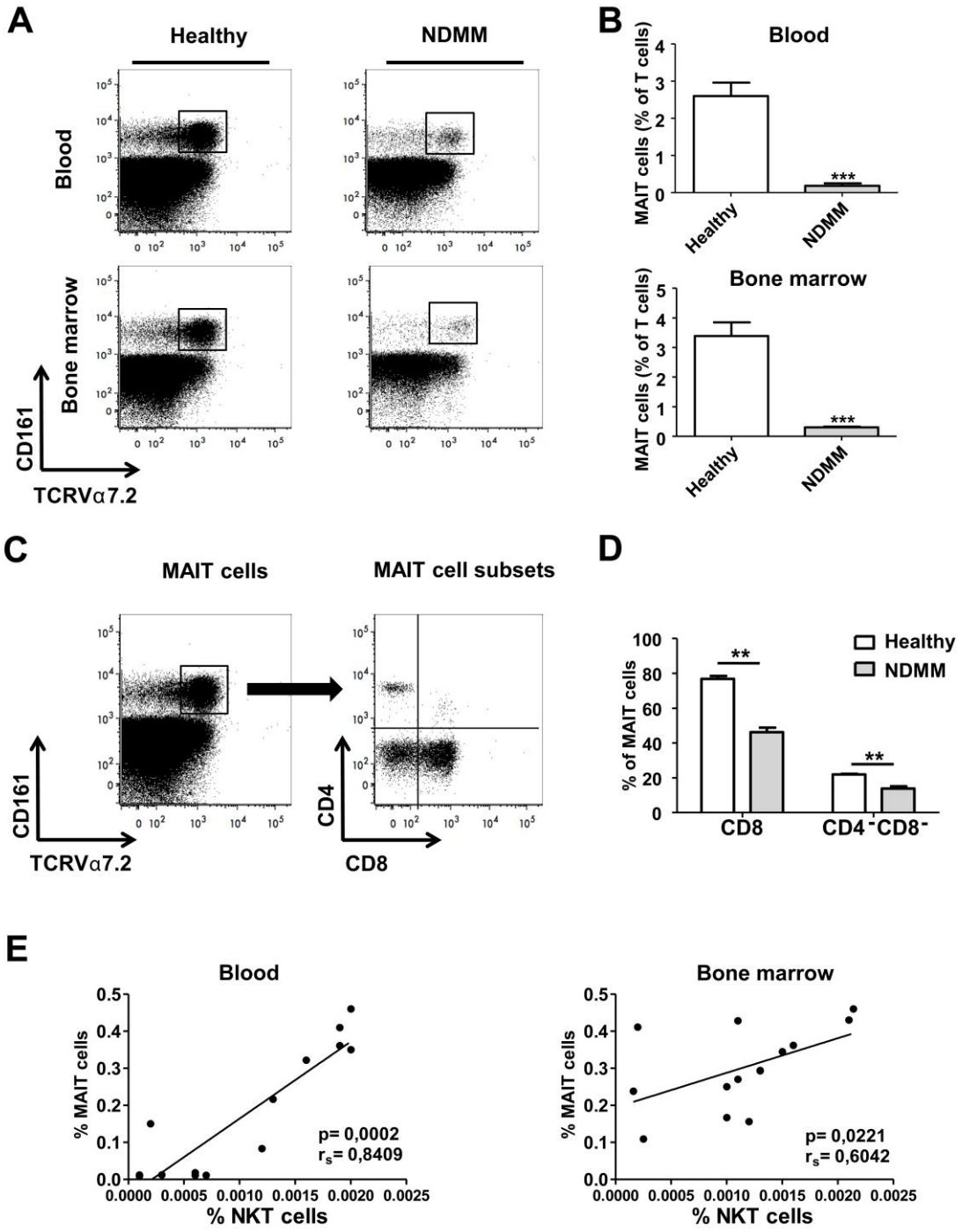

Figure 1: MAIT cell numbers are decreased in NDMM patients compared to healthy controls and correlate with decreased iNKT cell numbers. *A) Flow cytometry data plots illustrating the MAIT cell gating strategy in the peripheral blood and BM of healthy controls (n=14) and NDMM patients (n=14). B) MAIT cell percentages within peripheral blood and BM T cells in healthy controls and NDMM patients. C) Representative MAIT cell subsets as determined by flow cytometry and D) CD8+ and CD4$^-$CD8$^-$MAIT cell percentages within peripheral blood and BM T cells in healthy controls and NDMM patients. E) Spearman's correlation analysis between MAIT cell levels and NKT cell levels in blood and BM of NDMM patients. * p<0.05, ** p<0.01, *** p<0.001*

Additionally, similar decreases of iNKT cell numbers in MM could be observed (Figure S1A, S1B), in line with earlier reports[6]. Since MAIT and iNKT cells share a close lineage relationship we subsequently evaluated a potential relationship between both in NDMM patients, by analyzing the association between the MAIT cell levels and iNKT cell levels (Figure 1E). Spearman's correlation analyses revealed a significant correlation between MAIT cell percentages and the total iNKT cell levels in NDMM patients, suggesting that MAIT cell deficiency is linked to iNKT cell impairment.

Next, we evaluated the cytokine profile of MAIT cells in order to determine their functionality in NDMM. PBMCs from 7 NDMM patients and 7 healthy controls were stimulated for 4 hours in presence of PMA and ionomycin. Subsequently, expression levels of IFN-γ, IL-17, IL-22 and TNFα were examined by flow cytometry (Figure 2A). Expression of IFN-γ, IL-17, IL-22 and TNFα was absent on unstimulated MAIT cells of both patients and healthy individuals (data not shown). However, when stimulated, percentages of IFN-γ and TNFα positive MAIT cells were found to be significantly reduced in PBMCs derived from NDMM patients compared to healthy controls (Figure 2B). IL-17 and IL-22 MAIT cell expression levels were unchanged between NDMM patients and healthy donors (Figure 2B). As described in other studies, the majority of MAIT cells (80-90%) produce TNFα and IFN-γ compared to a minority (3% on average) of IL-17 and IL-22–producing MAIT cells[2]. Together, these data suggest that MAIT cells of NDMM patients have a disturbed Th1 function. This could be either due to reduced production of Th1 cytokines or polarization of their cytokine profile.

In order to assess whether iNKT cells have the capacity to activate MAIT cells, PBMCs from 7 NDMM patients and 7 healthy controls were incubated for 72 hours in presence or absence of α-GalCer, a strong glycolipid agonist for iNKT cells. Subsequently, CD69 and CD25 expression was determined in the MAIT cell population by flow cytometry (Figure. 2C). In healthy subjects, marked increases of $CD69^+$ and $CD25^+$ MAIT cells could clearly be observed after stimulation with α-GalCer in comparison with vehicle conditions (Figure 2D). In contrast, percentages of $CD69^+$ and $CD25^+$ MAIT cells were markedly reduced upon iNKT stimulation in NDMM patients. These findings suggest that a dysfunction of iNKT cells, MAIT cells or a combination of both could be responsible for a reduced activation of MAIT cells in NDMM patients as has also been observed in SLE[10]. Altogether this further supports the intriguing concept that dysfunctional iNKT – MAIT cell interactions can be involved in pathological conditions. Although underlying mechanisms of cross regulation between iNKT and MAIT cells are currently unknown, it can be anticipated that iNKT – MAIT cell communication is partly cytokine mediated. However, it should also be noted that iNKT cell activation leads to bystander stimulation of a broad range of downstream effector cells, including NK cells which could potentially also contribute to MAIT cell activation[1]. This will be the subject of future research.

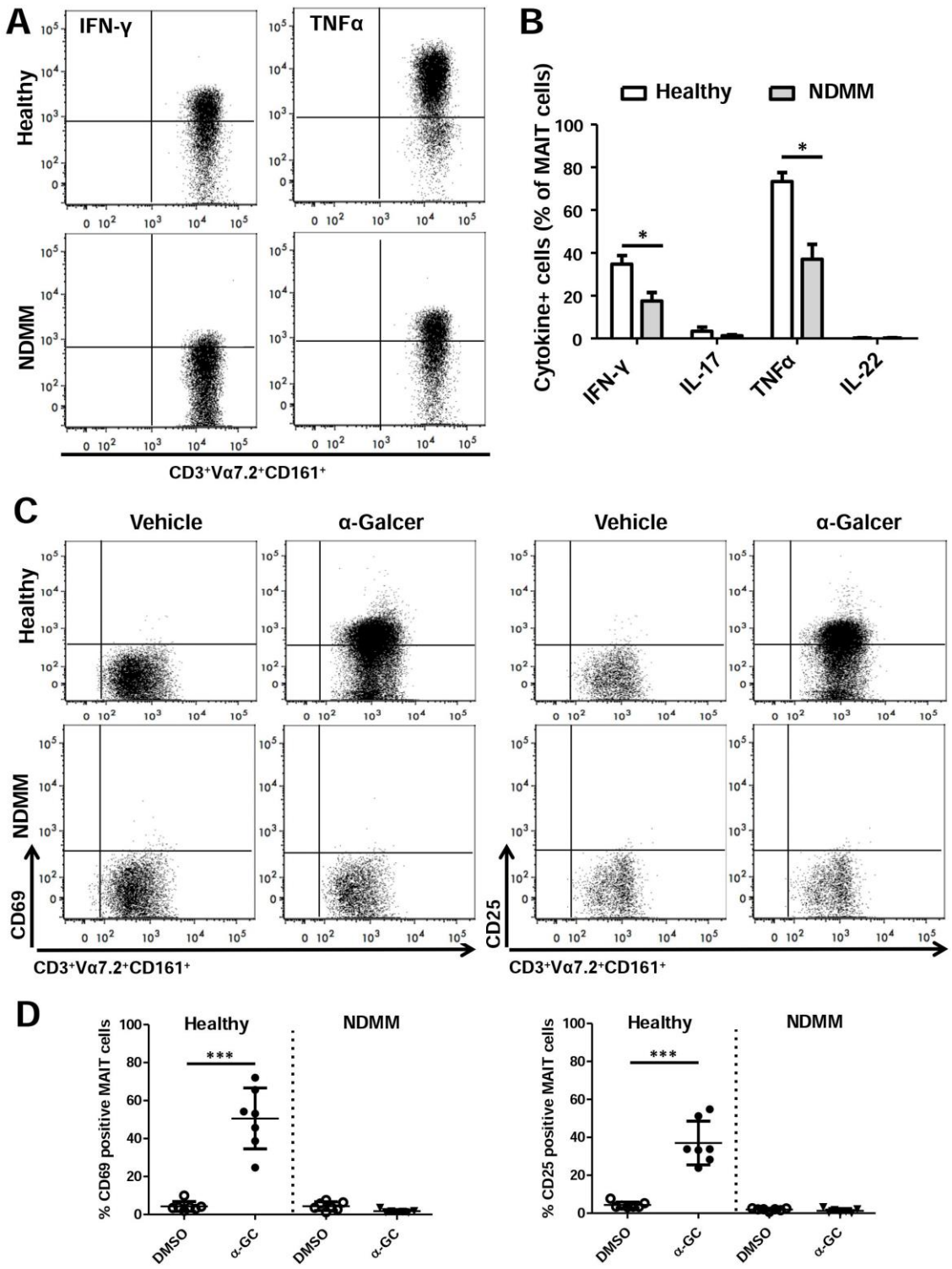

Figure 2: Impaired functionality of MAIT cells in NDMM patients compared to healthy controls. *A) Representative IFN-γ, IL-17, and TNFα expression levels in the MAIT cell population of healthy individuals (n=7) and NDMM patients (n=7) as determined by intracellular flow cytometry after stimulation with PMA and ionomycin. B) Percentages of IFN-γ, IL-17, IL-22 and TNFα levels produced by MAIT cells in response to PMA and ionomycin are illustrated. C) Flow cytometry data plots of CD69 and CD25 expression levels within the MAIT cell population of healthy donors (n=7) and NDMM patients (n=7) after 3 days of stimulation with or without α-GalCer (DMSO Vehicle) D) Percentage CD69 and CD25 positive NKT and MAIT cells after 3 days of stimulation with or without α-GalCer (Veh). DMSO (DMSO vehicle); Alpha-galactosyl ceramide (α-GalCer); * p<0.05, ** p<0.01, *** p<0.001*

PD-1 is a well-known target for immune checkpoint inhibition in cancer, as tumor cells are able to evade the immune system by PD-1 – PDL1/2 signaling. In conventional T cells, PD-1 is absent on naïve T cells but upregulated after T cell activation. Recent reports highlighted PD-1 and its ligand PD-L1/2 as being implicated in induction and maintenance of iNKT cell anergy[12–14]. We therefore investigated whether the observed iNKT and MAIT cell dysfunction was related to aberrant PD-1 expression that was determined by flow cytometry (Figure 3A). Interestingly, PD-1 levels were increased both on iNKT and MAIT cells in BM and PB of NDMM patients (n=14) as compared to healthy controls (n=14) (Figure 3B). Comparable results were observed in the 5T33MM murine model (Figure S2A). We next assessed the impact of PD-1 blockade on MAIT cell function in NDMM patients by performing co-culture experiments *in vitro*. PBMCs from 7 NDMM patients and 7 healthy controls were incubated for 72 hours in the presence or absence of α-GalCer with or without PD-1 blockade. Remarkably, activation of MAIT cells by α-GalCer stimulated iNKT was recovered in NDMM patients in presence of PD-1 blockade compared to the condition with α-GalCer and PD-1 block alone (Figure 3C). Similar immune activation by α-GalCer together with PD-1 blockade was found in the 5T33MM model (Figure S2B). Successful re-activation of MAIT cells by PD-1 blockade suggests that the PD-1 pathway is one of the contributors mediating MAIT cell dysfunction in MM and disturbing MAIT–iNKT immune interactions. Moreover, PD-1 blockade combined with α-GalCer stimulation led to a strong reduction in tumor load *in vivo* in the 5T33MM model (Figure S3).

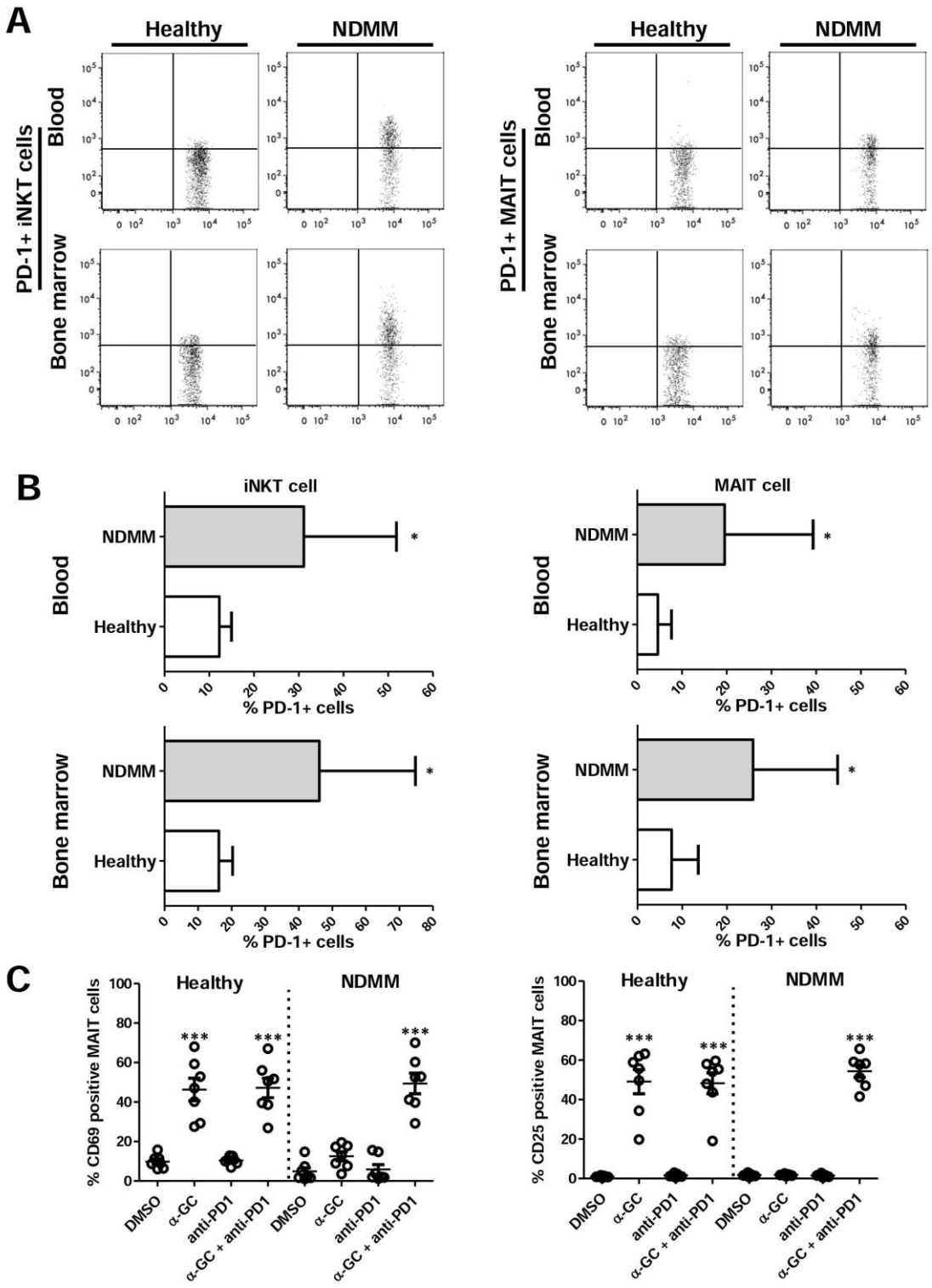

Figure 3: Increased PD-1 levels on MAIT and iNKT cells in NDMM patients and restoration of MAIT cell activation by iNKT stimulation and PD-1 blockade *in vitro*. *A) Flow cytometry data plots illustrating PD-1 expression levels on MAIT and iNKT cells in the peripheral blood and BM of healthy controls (n=14) and NDMM patients (n=14). B) Percentages PD-1 positive NKT and MAIT cells among peripheral blood and BM in healthy controls (n=14) and NDMM patients (n=14). C) Percentage CD69 and CD25 positive cells within the MAIT cell population of healthy donors (n=7) and NDMM patients (n=7) after 3 days of stimulation with or without α-GalCer (DMSO) and anti-PD-1. DMSO (DMSO vehicle); Alpha-galactosyl ceramide (α-GalCer); Programmed death 1 checkpoint molecule (PD-1) * p<0.05, ** p<0.01, *** p<0.001*

To the best of our knowledge, we are the first to demonstrate that MAIT cells are numerically and functionally impaired in NDMM patients (For an overview of the findings see Figure 4). Additionally, this was found to be linked with iNKT cell deficiency and elevated PD-1 levels. PD-1 blockade together with α-GalCer-stimulated iNKT cells rescued this deficiency and conferred tumor protection in the 5T33MM murine model. These results open doors for further studies and stimulates research to elucidate the exact role of MAIT cells in MM. Apart from evaluating MAIT cells in other MM patient subsets (MGUS, smoldering MM and relapse), long-term studies in large cohorts of patients will allow us to evaluate if these alterations can be linked to clinical outcome. Furthermore, it is of interest to evaluate MAIT and iNKT cell levels in anti-PD-1 treated MM patients. However, we believe that the impact of MAIT cells in the MM microenvironment as well as improvement of their effector functions via immune checkpoint blockade represents a relevant and attractive field for immunosurveillance and immunotherapy in MM. In line with Richter et al. demonstrating clinical regression after combining lenalidomide and iNKT cell stimulation in MM patients, we provide supplementary evidence for harnessing invariant T cells to prevent MM[15]. Therefore, targeting invariant T cells which simultaneously stimulate both innate and adaptive immunity, together with PD-1 blockade, might provide a more broad immune activation and could therefore give more advantageous results compared to current ongoing trials.

Overview findings

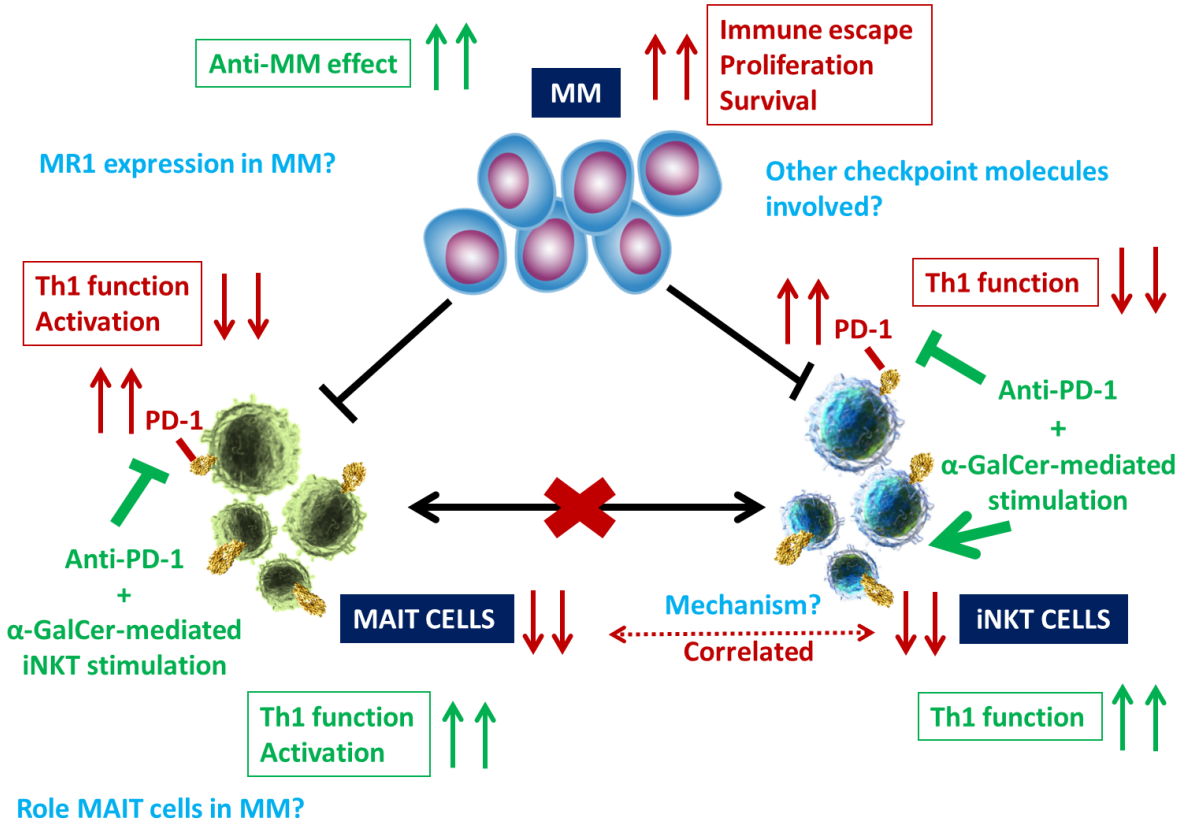

Figure 4: Overview general findings. The implication of checkpoint molecule PD-1, in mediating iNKT cell deficiency and deficiencies in mucosal-associated invariant T (MAIT) cells, another invariant T cell subset, was evaluated. Not only iNKT cells but also MAIT cells proved to be numerically and functionally impaired in MM (red boxes with downward arrows), a novel observation that hasn't been reported before. Moreover, we discovered MAIT cell deficiency to be correlated with reduced iNKT cell numbers in MM (red dotted arrow line). MAIT cells showed poor activation by α-GalCer-stimulated iNKT cells, illustrating a dysfunctional interaction between MAIT and iNKT cells (red cross). Remarkably, elevated PD-1 levels were found on both iNKT and MAIT cells (red upward arrow), contributing in the immune escape, proliferation and survival of MM (red upward arrows). Treatment with a combination of PD-1 blockade (green blocking arrow) and α-GalCer-mediated iNKT stimulation (green normal arrow) was able to significantly rescue the functionality of iNKT and MAIT cells and conferred superior tumor protection in MM (green boxes with upward arrows). However, several questions still remain unanswered (light blue) and will be the objective of future research. What is the role of MAIT cells in MM? What about MR1 expression in MM? What is the mechanism between the MAIT – iNKT interaction? Are there other checkpoint molecules involved in the immunodysfunction of MAIT and iNKT cells? *Alpha-Galactosylceramide (α-GalCer); Programmed death factor 1 (PD-1); Multiple myeloma (MM); T helper 1 function (Th1 function).*

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Supplementary material and methods

5T33MM mice

C57BL/KaLwRijHsd mice were purchased from Envigo (Horst, The Netherlands). Housing, maintenance and experiments were performed following regulations approved by the Ethical Committee for Animal Experiments, Vrije Universiteit Brussel (CEP n° 14_281_12). The 5TMM murine models have been previously described [1–3].

Myeloma Patients

After informed consent, BM and peripheral blood were collected from 14 untreated NDMM patients and age matched healthy controls. Patient characteristics are summarized in supplementary Table 1. The study was conducted in accordance with principles of the Declaration of Helsinki. Research was approved by the Ethic Board of UZ Brussel (B.U.N. 143201316382) and Tumourbank of Lille (CSTMT102).

Flow Cytometry and antibodies

Immunophenotyping was determined by flow cytometry using fluorochrome-conjugated monoclonal antibodies (mAbs). Analyses were performed using FlowJo software (Tree Star Inc., Ashland, OR).
Peripheral blood and BM samples from 14 healthy donors and 14 NDMM patients were collected in heparin-containing tubes. Human iNKT and MAIT cells were identified phenotypically as previously reported[4–7]. Mononuclear cells were obtained after Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich) and stained with PE-conjugated anti-human Vα24Jα18 TCR (6B11), PERCP Cy5.5-conjugated anti-CD161 (HP-3G10), Amcyan-conjugated anti-CD3ε (UCHT1), Pacific Blue-conjugated anti-CD4 (OKT4), Alexa Fluor 700-conjugated anti-CD8 (OKT8), PE-eFluor 610-conjungated anti-PD-1 (J43) (all eBioscience, San Diego, CA), Brilliant Violet 605-conjungated anti-TCRVα7.2 (3C10, Biolegend, San Diego, CA) and acquired on a FACSLSRII (Becton Dickinson). Intracellular cytokine flow cytometry was performed to determine the expression levels of IFN-γ, IL-17, TNFα and IL-22 in MAIT cells of NDMM patients and healthy controls. MAIT cells were stained with PERCP Cy5.5-conjugated anti-IFN-γ, PERCP Cy5.5-conjugated anti-IL-17, PE-conjugated anti-TNFα, and PE-conjugated anti-IL-22 (all eBioscience) and analyzed by flow cytometry (FACSCanto II; Becton Dickinson). For the analysis of activation markers, APC-conjugated CD69 (FN50) and PE-Cy7-conjugated CD25 mAbs (BC96, both eBioscience) were used.

Murine liver, blood, spleen were isolated as previously described[1,8]. BM cells were isolated as described earlier in the article. Lymphocytes were isolated and resuspended in staining buffer containing anti-Fcγ Receptor type II/type III monoclonal antibodies (Miltenyi Biotec). Cells were

stained with PE-labelled CD1d/α-GalCer tetramer, FITC-conjugated anti-279 (PD-1, J43), APC-Cy7-conjugated anti-TCRβ (H57-597), PE-Cy7-conjugated anti-CD3 (145-2C11), Pacific Blue-conjugated anti-NK1.1 (PK136, all eBioscience), Amcyan-conjugated anti-CD4 (RMA4-5) and 7-AAD (Both Beckton Dickinson) and living cells were acquired on a FACSCantoII (BD Biosciences) flow cytometer.

Functional MAIT cell assays

PBMCs (1,5 x10^6/ well) were incubated in complete RPMI 1640 with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS (Life technologies BRL), stimulated with Phorbol-Myristate-Acetate (PMA, 100 ng/ml; Sigma-Aldrich), ionomycin (1 µM; Sigma-Aldrich) and brefeldin A (GolgiPlug; 10 µg /ml; BD Biosciences) for 4 hours and subsequently stained for MAIT markers. After fixation in 4% paraformaldehyde and permeabilization with Perm/Wash solution (BD Biosciences), MAIT cells were intracellularly stained for IFN-γ, IL-17, TNFα and IL-22 and analyzed by flow cytometry.

PBMCs (1,5 x 10^6/ well) were stimulated with α-GalCer (100 ng/ml; Department Chemistry; Ghent University) or 0,1% DMSO (vehicle) and incubated with or without anti-human anti-PD-1 Ab (5 µg/ml, eBioscience) for 3 days in the presence of IL-2 (100 U/ml, Roche). After 72 hours, PBMCs were stained and analyzed by flow cytometry for MAIT cells and iNKT cells markers next to the activation markers CD69 and CD25.

NKTMM co-culture assay

Isolation of 5T33MMvv, 5T2MM, iNKT and BM cells were performed as previously described[1,8,9,10]. 5x10^4 5T33MMvv / 5T33MMvt / 5T2MM cells, 5x10^4 iNKT cells, 10^5 α-GalCer loaded or vehicle loaded DCs were co-cultured in triplicate in presence or absence of murine anti-PD-1 Ab (5 µg/ml, Bioceros, The Netherlands). After 24 hours supernatants of co-cultures were collected for IFN-γ measurements by ELISA (eBioscience), following manufacturer's instructions.

***In vivo* treatment with α-GalCer and anti-PD-1 in MM**

Naive C57BL/KaLwRijHsd mice were i.v. injected with 5 x 105 5T33MM cells and i.p. injected with 200 µg murine anti-PD-1 Ab and 2 µg α-GalCer. α-GalCer injections were repeated weekly (day 6, day 13) and anti-PD-1 Ab was given twice a week. Serum IFN-γ levels were determined by ELISA, 16 hours after i.p. α-GalCer administration (day 0 and day 6). Mice were sacrificed when first signs of morbidity appeared (in general at day 21). Serum M–spike levels were determined by protein electrophoresis and cytosmears were processed to evaluate BM tumor load.

Statistics

Results are expressed as mean ± SEM. Spearman's Correlation, one-way ANOVA with Bonferroni correction and Mann-Whitney U-tests were used to address statistical significance.

Supplementary figures

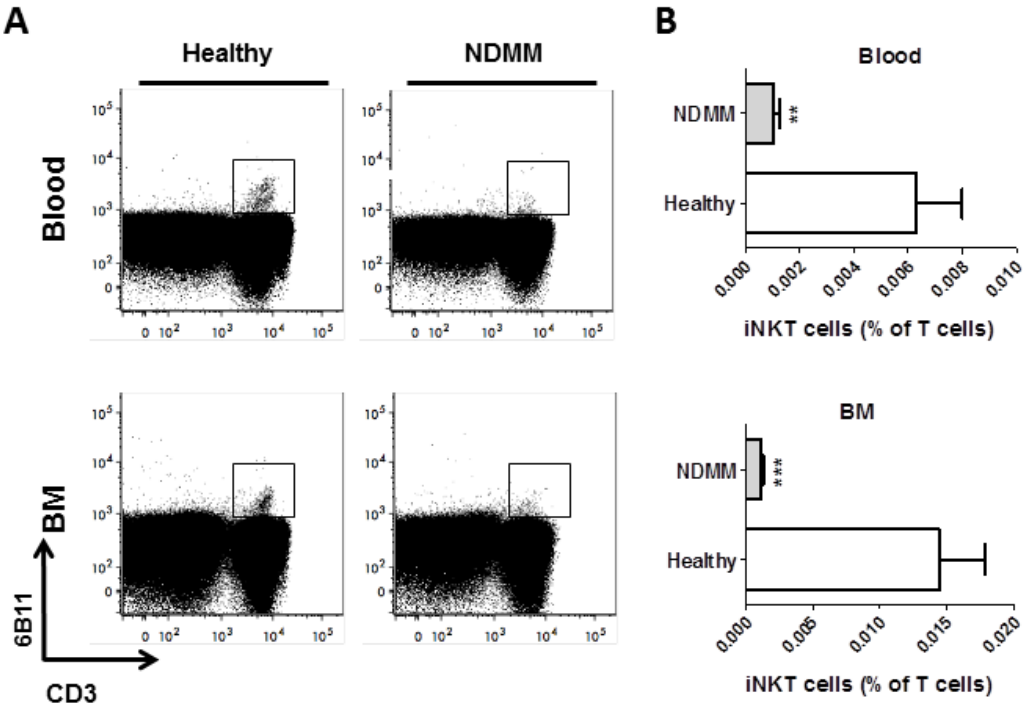

Figure S1: Decreased iNKT cell numbers in NDMM patients compared to healthy controls. *A) Flow cytometry data plots illustrating the iNKT cell gating strategy in the peripheral blood and BM of healthy controls (n=14) and NDMM patients (n=14). B) iNKT cell percentages within peripheral blood and BM T cells in healthy controls and NDMM patients. * p<0.05, ** p<0.01, *** p<0.001*

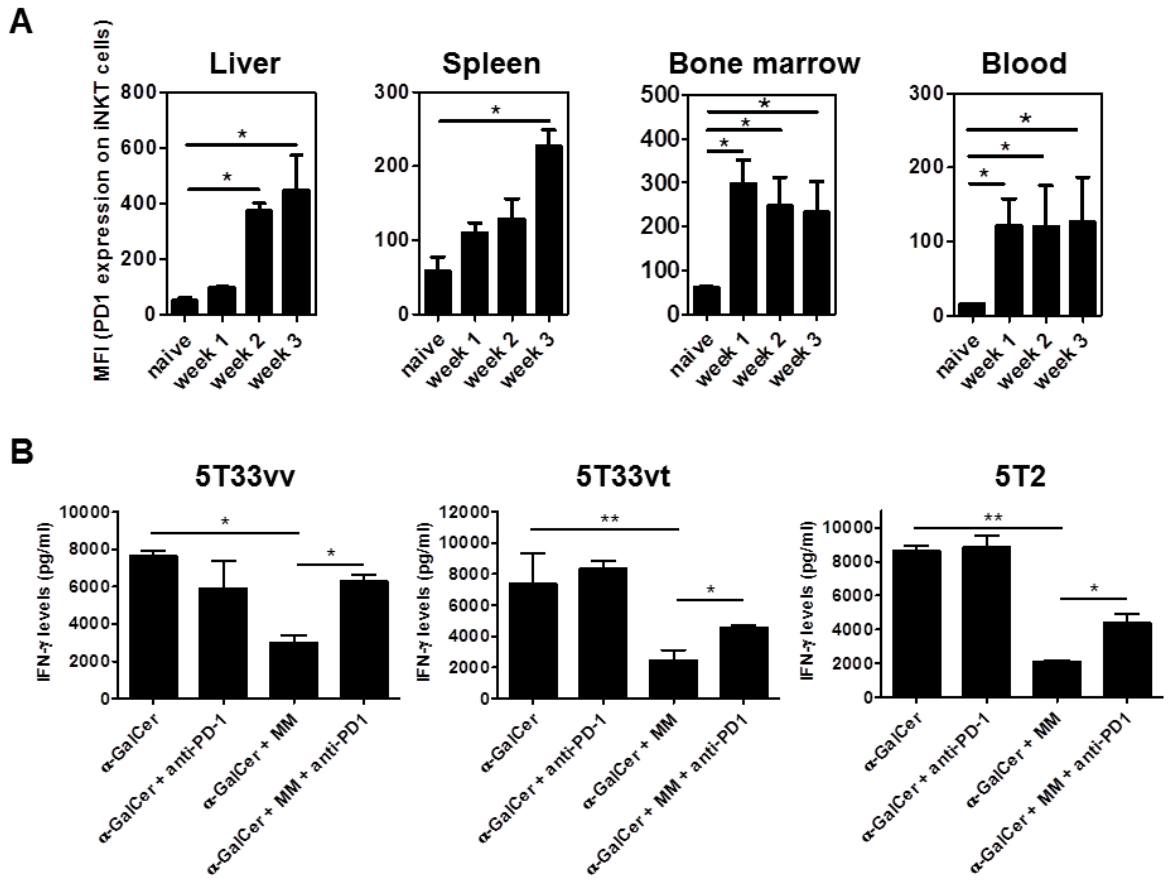

Figure S2: Increased PD-1 expression on iNKT cells in the 5T33MM murine model and restoration of iNKT functionality by PD-1 blockade *in vitro*. *A) Percentages PD-1 positive NKT cells in liver, spleen, BM, blood of the 5T33MM murine model. B) Mean IFN-γ levels of murine iNKT co-culture experiments are illustrated. The experiment was performed with 5T33vv, 5T33vt and 5T2 MM cells. Respective conditions are iNKT cells + α-GalCer-loaded DC's; iNKT cells + α-GC-loaded DC's + anti-PD-1; iNKT cells + α-GC-loaded DC's + MM cells; iNKT cells + α-GC-loaded DC's + MM cells + anti-PD-1. Vivo (vv); Vitro (vt); Alpha-Galactosylceramide (α-GalCer); Multiple myeloma cells (MM). * p<0.05, ** p<0.01, *** p<0.001*

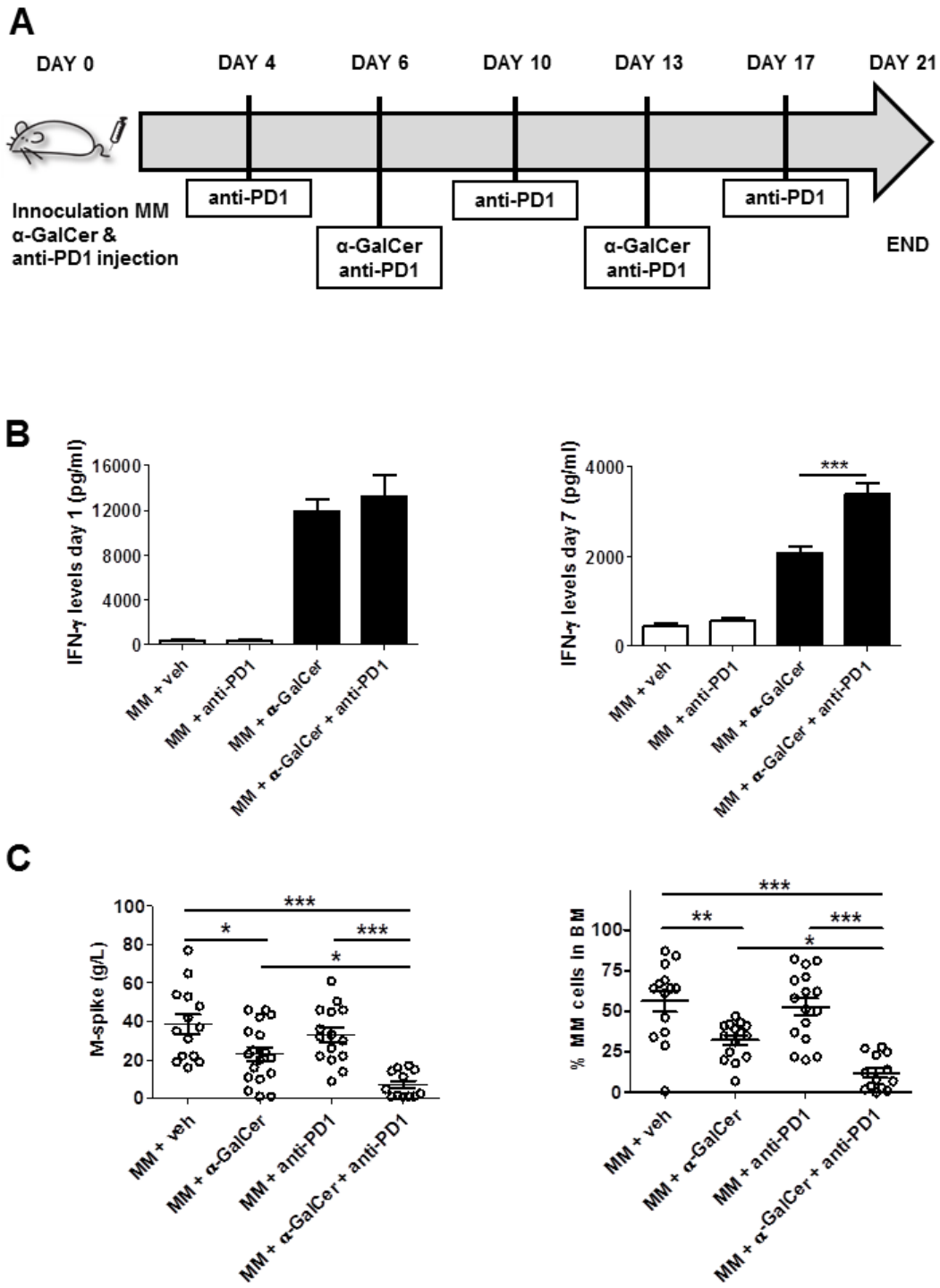

Figure S3: *In vivo* treatment of PD-1 blockade and α-GalCer restored tumor immunity in the 5T33MM murine model. *A) Schematic overview of the experiment. C57BL/KaLwRij mice were inoculated together with 2µg α-GalCer and 200µg anti-PD-1 at day 0. α-GalCer was re-injected weekly at day 6 and day 13. Injections with anti-PD-1 were given twice a week. Treatment groups were MM + veh (n=14), MM + anti-PD-1 (n=16), MM + α-GalCer (n=15), MM + α-GalCer + anti-PD-1 (n=13). B) Serum M-spike levels and percentage malignant plasma cell in the BM. C) IFN-γ levels in the serum 16h after the first stimulation and second stimulation (one week later) with α-GalCer. * p<0.05, ** p<0.01, *** p<0.001*

Supplementary table 1: Characteristics healthy controls and NDMM patients

	Number	Mean	Range
Control individuals (n= 14)			
Age (years)		62	54-76
Gender (M/F)	8/6		
Newly Diagnosed multiple myeloma (n= 14)			
Age (years)		70	57-81
Gender (M/F)	8/6		
M-component type			
IgG	10		
IgA	3		
IgM	1		
IgD	-		
Light chain type			
kappa	7		
lambda	7		
ISS Stage[1]			
Stage I	4		
Stage II	6		
Stage III	4		
Adverse cytogenetics			
13q	5		
t(11,14)	9		
17p	2		

[1]**ISS: International Staging System**

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Chapter VI

General discussion and future perspectives

Treatment of multiple myeloma (MM) changed considerably over the last two decades with the introduction of novel therapeutic agents such as autologous stem cell transplantation, proteasome inhibitors and immunomodulatory drugs, as well as combination therapies, significantly improving the survival outcomes for patients[1,2]. Although few remain in long-term remission, the majority of MM patients still relapses and eventually dies from the disease, usually due to a resistant clone. It is well established today that MM is associated with a progressive immune dysregulation, resulting in a loss of immunosurveillance and subsequently promoting tolerance and tumor progression[2]. Although MM is primarily a disease of the B cell lineage, the T cell compartment is also strongly affected[3]. A significant loss in T cells of the CD4, CD8, iNKT and MAIT cell subsets is reported, while on the other hand a recruitment and a concomitant rise in suppressor cells, including Treg cells, TAMs and MDSCs is observed[4–10]. MM immune dysregulation also disturbs other aspects of the immune system as well, directly affecting antigen presentation and the upregulation of inhibitory antigens and checkpoint molecules that promotes immune escape and growth advantage for malignant clones[11,12]. Therefore, there is a crucial need to continue preclinical research and to find new therapeutic strategies to target the immune system in the treatment for MM. Currently, immunotherapy is revolutionizing the landscape of cancer treatment and has the potential to improve patient outcomes significantly in the future. Since the regulatory approval of ipilimumab in 2011, the field of cancer immunotherapy has experienced a renaissance. This success is based on progress in both preclinical and clinical science, including the development of new immunotherapeutic strategies. Immunotherapy mobilizes and boosts the patient's own immune system through various approaches that utilize adaptive or innate immunity, as previously highlighted in the general introduction (see section 4.4, Chapter I).

In this thesis we primarily focused on iNKT cell immunology in MM. iNKT cells represent a distinct population of T lymphocytes, with features of both conventional T cells as well as NK cells. As a result of their unique ability to recognize endogenous lipid antigens in a CD1d-dependent manner, iNKT cells have a constitutive memory phenotype and moreover, as being one of the first responders of the immune system, are capable of rapidly responding to stimulation by producing a broad range of cytokines[13]. In addition, through direct interactions, in particular via CD1d and CD40L-CD40 signaling, as well as indirect interactions with other immune cells, iNKT cells are capable of maturing DCs and activating B cells, and thus are crucial in enhancing antigen-specific B-and T-cell responses[14]. Their ability to bridge the innate and adaptive immunity and their capacity to modify the immune microenvironment influences the capability of the host to control tumor growth, making them an important population to be harnessed in the clinic for the development of anti-cancer therapeutics. Indeed, one of the advantages of iNKT cell-targeted anti-tumor immunotherapy is that, compared with current anti-cancer immunotherapies (that only target one arm of the immune system), a

variety of anti-tumor immune effector cells, such as NK cells, B cells, neutrophils, DCs, $CD8^+$ and $CD4^+$ T cells, can be targeted simultaneously. Moreover, another benefit of iNKT cell-targeted anti-tumor immunotherapy over a more traditional T cell-based immunotherapy is the competency of iNKT cells to target both MHC-negative and MHC-positive cancer cells[15]. The importance of iNKT cells in tumor immunosurveillance is further emphasized by the observation of reduced iNKT cell numbers and an impaired function in a large number of cancers, including a broad range of solid malignancies but also in hematological malignancies, as reported by us in MM[5,16–18]. The use of iNKT cell deficient mice and the identification of strong iNKT cell glycolipid agonists, such as α-galactosylceramide (α-GalCer) and its analogues, have led to the development of synthetic lipids that have shown potential in vaccination and treatment of cancers[19].

Two main iNKT cell-directed therapeutic options have been exploited thus far, including administration of iNKT cell-activating ligands (all human studies described to date have used α-GalCer), administration of antigen presenting cells (APCs) pulsed with α-GalCer, transfer of ex vivo-expanded and/or activated iNKT cells[14]. The use of α-GalCer and other glycolipids has engendered preclinical success in mice, leading to multiple clinical trials in patients with anal cancer, renal cell cancer, MM, non-small cell lung cancer and head and neck squamous cell carcinoma (see Table 1, Chapter II). Despite the benefits for patients looking promising, results remain disappointing and far from effective since iNKT cell-based immunotherapy is associated with some challenges limiting its efficacy[20]. Of note, the frequency of iNKT cells is much lower in humans compared to mice and additionally humans also have a heterogeneous range of iNKT cell numbers, possibly contributing to the heterogeneity in clinical responses[21–23]. Although we know that low numbers of iNKT cells are associated with poor prognosis in autoimmune or malignant disease, it remains unknown if high or low numbers of iNKT cells are an important biomarker for the outcome of an iNKT cell-based therapy[20,24,25]. Studies aimed at enhancing iNKT cell-mediated anti-tumor immunity have shown that the use of α-GalCer leads to the potent stimulation of iNKT cells but unfortunately results in iNKT cell over-activation and an exhausted state[26–28]. This means they are sensitive to anergy which is a property that, next of being exploited by cancer cells as an immune evasion mechanism, could be an explanation for the lack of therapeutic success. Moreover, suboptimal activation due to an uncontrolled distribution of α-GalCer also potentially can be a problem hindering the beneficial effects of an iNKT cell-based immunotherapy[29,30]. Furthermore, the existence of different NKT (sub)populations, their adaptable reactivity against ligand agonists and the different APCs involved in the antigen presentation makes iNKT immunotherapy a more complex story[13,31,32]. Finally, our knowledge of the presence of endogenous ligands is still very limited hampering our true understanding of iNKT cell biology[33]. In order to overcome the different challenges described above

and to enable a correct manipulation of iNKT cells in anti-tumor therapy, it is of great interest to further enlarge our understanding about iNKT cell biology and the factors activating and regulating these cells or making them deficient in pathologic conditions and cancers such as MM.

In this PhD project we aimed to unmask the mechanisms responsible for iNKT cell immune dysfunction in MM. By subsequently rescuing iNKT immune impairment anti-MM effects could be generated (Chapter IV and V):

In chapter IV, we investigated the curious interplay between leptin receptor signaling and iNKT cell-mediated anti-tumor immunity in MM. Adipocytes were recently identified as an immunosuppressive population in the BM niche and since an age-related increased adipose tissue mass in the BM is correlated with an elevated risk for MM development, we hypothesized this could be the result of a defective anti-tumor immunity mediated by adipokines such as leptin[34]. Our results revealed that leptin controls the iNKT cell function and modulates iNKT cell threshold activation, subsequently preventing long term anti-tumor effects in MM. Blockade of leptin receptor signaling with an in-house made antagonist in combination with iNKT stimulation was able to counteract the immunosuppression of iNKT cells and resulted in superior tumor protection as a result of an alleviation of iNKT cell anergy. Interestingly, these findings may let us think that the leptin-leptin receptor axis acts like an immune checkpoint barrier, enhancing immune suppression and evasion in pathologic conditions with elevated leptin/ leptin receptor levels, such as detected in MM. Being confronted in our research with iNKT cell anergy induced by MM cells themselves and by repeated injections of α-GalCer we further elaborated on the subject. In chapter V we found that, in MM, iNKT cells showed a marked increase in programmed death-1 (PD-1) expression, hampering the beneficial and/ or long lasting effects of an iNKT cell-mediated treatment. The use of an anti-PD-1 checkpoint inhibitor restored their anti-tumor properties and subsequently combining PD-1 blockade with α-GalCer stimulation led to a superior anti-MM effect. Being interested in the innate-like T cells in general, we also made another observation in this study. Never being investigated before in MM, we discovered MAIT cells to be numerically and functionally impaired in MM as well. Moreover, we demonstrated that their deficiency was found to be correlated with iNKT cell deficiency. A dysregulation of MAIT cells in cancer and especially in non-mucosa-associated cancer is gaining interest in the field and is becoming reported by several researchers[35–42]. Also on MAIT cells we found PD-1 levels to be high in MM. Blocking PD-1 together with iNKT cell stimulation partially restored the MAIT cell functionality. These observations, next to demonstrating the importance of blocking anergy to obtain beneficial therapeutic outcomes, also further highlights one of the advantages of iNKT cell immunotherapy described above, being the ability to activate different other

effector cells of the immune system. However, the role and function of MAIT cells in MM remains unknown and certainly needs investigation in the future.

We can state that anergy affects the efficacy of an iNKT cell-based immunotherapy in cancers, such as MM, in two ways: On the one hand, anergy is used as a switch-off mechanism for the immune system by the cancer itself to enhance immune escape[43]. On the other hand, studies aimed at enhancing iNKT cell-mediated anti-tumor immunity have shown that multiple use of α-GalCer leads to the potent over-activation of iNKT cells but results in a functionally blunted state because of anergy induction[26,28,44]. In the next paragraph, we will discuss and suggest some future approaches to overcome anergy hampering the success of an iNKT cell-based immunotherapy in cancer, and thus also in MM.

1. As a first approach to improve iNKT cell-based immunotherapy we suggest *the targeting of immune checkpoint proteins.* The scientific turning point for cancer immunotherapy came with the understanding that T cell immune responses are controlled through on and off switches, so called 'immune checkpoints' that protect the body from possibly damaging immune responses. Immune checkpoint pathways, such as PD-1 and CTLA-4 help to maintain immune equilibrium in health but are upregulated in the presence of malignancy, fostering a state of immune tolerance. The upregulation of these negative costimulatory signals induces an anergic state of T cells, blunting T cell activation and expansion, and blocking T cell–mediated killing of cancer cells[45]. PD-1 ligands, PD-L1 or PD-L2, are frequently expressed on tumor cells and can further minimize the immune response. Remarkable results with different PD-1 (nivolumab, pidilizumab and pembrolizumab) and PD-L1 (atezolizumab and avelumab) inhibitors indicated promising clinical efficacy and a well-tolerated toxicity profile in various cancer patients with melanoma, non-small cell lung cancer, kidney cancer and head and neck cancers[46,47,48–50]. Also in hematological malignancies immune checkpoint blockade looks a promising strategy to revert anergy of the immune system[51]. PD-L1 is expressed in a variety of hematological malignancies such as leukemia and peripheral T-cell lymphoma and has been correlated to poor prognosis[52–55]. In MM, studies have demonstrated the expression of PD-L1 on MM cells and immune cells and expression of PD-1 on different T cell subsets and natural killer cells within the MM microenvironment[56–58]. In chapter V we revealed that the majority of iNKT and MAIT cells of MM patients expressed PD-1 and that PD-1 blockade ameliorated the iNKT cell-based immunotherapy. This is in line with others who have shown that PD-1 possesses a significant impact in α-GalCer-induced iNKT cell anergy[59]. Namely, iNKT cells from α-GalCer-treated PD-1-deficient mice do not show an anergic response to α-GalCer-stimulation and α-GalCer-induced anergy can even be prevented in WT mice by blocking the PD-1/PD-L interaction by using anti-PD-1 or a combination of anti-PD-L1 and anti-PDL-2 mAbs[59]. In a mouse B16 melanoma model, the anti-metastatic activities of

α-GalCer-activated iNKT cells could be restored by abrogating the PD-1/PD-L1 interaction during initial α-GalCer treatment[59]. In the future, we would like to investigate the blocking of PD-L1 and PD-L2. Based on the known interactions of the PD-1 ligands, it is theoretically possible that the PD-1 antibody has a distinct biological activity from PD-ligand antibodies. A PD-1 antibody blocks PD-1 interaction with both PD-L1 and PD-L2 but not the interaction between PD-L1 and CD80 while most PD-L1 antibodies block the interaction between PDL1 and CD80 and between PD-L1 and PD-1 but not with PD-L2[60]. Thus, it is possible that using a combination of both would ameliorate the therapeutic effects even more.

However, blockade of the CTLA-4 and PD-1/PD-L1 pathways only represents the tip of the iceberg in the realm of potential immune checkpoint targets. Ongoing studies on regulation of immune responses have led to the identification of multiple other immunologic checkpoint pathways namely lymphocyte activation gene 3 protein (LAG-3), T cell immunoglobulin mucin receptor 3 (TIM-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT) and V-domain immunoglobulin (Ig)-containing suppressor of T cell activation (VISTA)[61,62]. Preclinical studies also indicated the possibility of simultaneous co-expression of different checkpoint molecules, such as LAG-3 or TIM-3 being co-expressed with PD-1 on tumor-infiltrating lymphocytes[63,64]. This could lead, without simultaneous targeting, to a suboptimal response to immunotherapy. Regarding the improvement of the iNKT cell-based immunotherapeutic approach it should therefore be of great value to further investigate these different newly identified checkpoint proteins and their relation with iNKT cell anergy. Moreover, we are convinced that the list of immune checkpoint molecules is still far from being complete. In our search for mechanisms contributing to iNKT cell dysfunction in MM, we discovered a new immune checkpoint therapeutic approach that alleviated iNKT cell anergy and prolonged the activation of α-GalCer-stimulated iNKT cells: the targeting of the leptin-leptin receptor axis mediated by the immunosuppressive adipocytes in the MM microenvironment (Chapter V). However, the mechanisms behind leptin-induced anergy in MM are still unknown and should clearly be the subject of further investigation. The use of a conditional leptin and/or leptin receptor knock out in MM in future research could help elucidate this part of the story. Moreover, understanding the contribution of leptin an its receptor in mediating anergy could also be beneficial for the treatment of a large variety of other cancers such as breast, pancreas, lung, thyroid, endometrial and gastrointestinal cancer, where the leptin-leptin receptor axis also clearly plays a role in the pathology[65,66]. Given the results achieved within this PhD project, it would be of interest to study whether simultaneous blockade of the leptin receptor and PD-1 together with the stimulation of iNKT cells with α-GalCer or another potent and improved analogue (see section 4 below) would initiate a synergistic antI-tumor response in MM.

2. In addition to inhibitory checkpoint molecules, it has become clear that *co-stimulatory molecules* are also involved in T cell exhaustion. Co-stimulatory molecules can be subdivided in the ICOS/ICOSL family, the B7/CD28 family, the CD40/CD40L family and tumor necrosis factor (TNF)/tumor necrosis factor receptor family, including molecules such as OX40 (CD134) and 4-1BB (CD137)[67]. All of the above mentioned molecules were demonstrated to be involved in iNKT cell co-stimulation as reviewed in [68]. Desensitization of co-stimulatory pathway signaling through the loss of adaptor molecules can result in anergy and allow tumors to evade the immune system[69]. Therefore it could be relevant to exploit the potential beneficial role of co-stimulation, eventually together with immune checkpoint blockade to reverse exhaustion by using agonistic antibodies to positive co-stimulatory pathways, and by this way improve iNKT cell based-immunotherapy.

3. Target altered gene expression. Recent reports have demonstrated that induction of E3 ubiquitin ligases, including Casitas B cell lymphoma-b (CBl-b), Itch, Deltex-1, and gene related to anergy in lymphocytes (GRAIL), is required to induce and maintain T cell anergy, thus implicating the protein ubiquitination pathway in development of immune tolerance[70–73]. The E3 ubiquitin ligase GRAIL functions via the ubiquitination and degradation of actin cytoskeleton-associated proteins, in particular Arp2/3-5 and coronin 1A, thereby maintaining the unresponsive state of anergic T cells[74]. Wu et al. demonstrated the implication of an impaired upregulation of GRAIL expression in α-GalCer-induced anergy of iNKT cells[75]. Deltex-1 is a transcription target of the nuclear factor of activated T cells (NFAT). As a result of an upregulation of Calcium and NFAT signaling, Deltex-1 expression increases during T cell anergy. Subsequently, the expression of two anergy-associated molecules: growth arrest and DNA-damage-inducible 45 β (Gadd45β) and CBl-b is upregulated[73]. The E3 ligase Itch was currently only reported to mediate tolerogenic/ anergic processes in Th2 cells via mitogen and extracellular kinase kinase 1 (MEKK1) - JNK signaling. Both the role of GRAIL, Deltex-1 and Itch in α-GalCer-induced iNKT cell anergy is till now unclear, but interesting to further look into. Especially since CBl-b was recently demonstrated to be directly involved in α-GalCer-induced iNKT cell anergy[76]. Kojo et al. namely observed that α-GalCer experienced iNKT cells had an enhanced CBl-b expression, stimulating the monoubiquitination of Caspase recruitment domain-containing protein 11 (CARMA1) and consequently affecting IFN-γ production through the NF-κB activation pathway. Interestingly, CBl-b-deficiency largely rescued reduced IFN-γ production, while CARMA1 deficiency drastically reduced the iNKT cell IFN-γ production in response to α-GalCer treatment[76]. These observations may indicate that α-GalCer experienced iNKT cells may have distinct regulatory mechanisms of hyporesponsiveness and impaired cytokine production. Furthermore, Safford et al. identified the zinc finger transcription factor early growth response gene 2 (Egr2) and Egr3, as being associated with an increase in CBl-b[77]. Consequently, targeting CBl-b, Egr2 and/ or Egr 3 by small molecule or peptide

antagonists and CARMA1 by agonists could clearly be of benefit in a combinatory administration with α-GalCer to prevent iNKT cell anergy.

Additionally, tuberous sclerosis 1 (TSC1), a negative regulator of the mTOR signaling was identified by Wu and colleagues to also play a crucial role in iNKT cell anergy as well[75]. Deficiency of TSC1 in iNKT cells resulted in resistance to α-GalCer-induced anergy. In response to secondary antigen stimulation, an increased expansion and cytokine production by iNKT cells was observed. Moreover, TSC1 appeared to be correlated with an impaired upregulation of PD-1, Egr2, and GRAIL. Interestingly, TSC1-deficient iNKT cells displayed enhanced anti-tumor immunity in a melanoma lung metastasis model[75]. Targeting TSC1 looks very promising as a strategy for improving α-GalCer-induced anergy.

To conclude, Stephen et al. also identified a new mechanism potentially mediating T cell exhaustion[78]. Briefly, they found that TCR activation induced increases in chromatin organizer special AT-rich sequence-binding protein-1 (Satb1) expression. Focusing on the immunosuppressive cytokine TGF-β, present in high amounts in tumor microenvironments such as MM, they observed that TGF-β reduces Satb1 levels and elicited higher PD-1 expression levels in activated T cells[78]. This suggests that Satb1 contributes to restrain PD-1 expression. Understanding the full spectrum of activities of the genomic organizer Satb1 in the process of T cell exhaustion could be required for further advances in preventing tumor-mediated T cell exhaustion and could also be an interesting approach to ameliorate iNKT cell-based immunotherapy.

4. *iNKT cell agonists or α-GalCer derivates.* The identification and optimization of iNKT cell agonists or α-GalCer derivates which can superiorly promote Th1 anti-tumor responses without inducing iNKT cell anergy are also of high priority. The search for efficient iNKT agonists with functional differences, including optimized cytokine induction profiles, compared to α-GalCer should help to reduce anergy in the future and is currently intensively investigated (reviewed in[79]). For example, the C-glycoside analogue of α-GalCer (α-C-GalCer) elicits a strong Th1 cytokine iNKT cell response and increased the immunogenicity by as much as 1000-fold[80]. The novel 7DW8–5 analogue of α-GalCer also showed to be at least 100-fold more active at stimulating human and mouse iNKT cells[81]. Recently Huang et al. demonstrated that phenyl-glycolipids compared to α-GalCer could restimulate iNKT cells to secrete Th1/Th2 cytokines upon second exposure without inducing anergy[44]. Phenyl-glycolipids displayed greater binding avidity and stability for iNKT T cell receptors (TCRs) when complexed with CD1d, driving higher TCR signaling, Th1 polarization and greater anti-cancer potency[44]. Previous work performed by colleague Aspeslagh S. showed that NU-α-GalCer, is a much more potent glycolipid than α-GalCer in terms of IFN-γ and IL-12 production and thus enhanced a more favorable Th1 polarization of iNKT cells[82]. Additionally, she found PyrC-α-GalCer (carbamate

glycolipid) to induce a 12-fold increase of IL-12 production compared to α-GalCer and a 4-fold increase compared to NU-α-GalCer, as well as superior anti-tumor responses in the B16 melanoma model[83]. The results of these altered lipids represent an exciting new opportunity to optimize therapeutic approaches as performed in this PhD project.

5. *Loading of α-GalCer onto new delivery systems.* A last approach that would be of great value to investigate and could lead to less anergy, allowing multiple injections and therefore a more prolonged response could be the loading of α-GalCer onto new delivery systems, such as liposomes , bacterial membranes, fusion proteins and (d)exosomes[29,30]. This could overcome a suboptimal activation due to an uncontrolled distribution of α-GalCer, what explains the low benefits of the currently used activation systems. Several experimental approaches can be and are currently developed to assist in targeting the delivery of α-GalCer to specific sites for more directed activation of iNKT cells. Nakamura et al. incorporated α-GalCer into stearylated octaarginine-modified liposomes (R8-Lip), a delivery system developed for vaccines[84,85]. Making use of α-GalCer/R8-Liposomes, α-GalCer presentation on CD1d in APCs was enhanced resulting in an expansion of iNKT cells. Moreover, positive therapeutic effects were obtained against highly malignant B16 melanoma cells. They showed R8-Lip to be a potent delivery system and demonstrated that size control and R8-modification in liposomal construction are promising techniques for achieving an efficient systemic administration of α-GalCer[84]. Similar results were obtained with unmethylated cytosine-phosphorothioate-guanine oligodeoxynucleotides encapsulated in cationic liposomes (CpG-liposomes)[86]. However, the effect on iNKT cell anergy was not yet investigated, similar liposome approaches are worthy to be further investigated. Another relatively simple and effective delivery method which could ameliorate both iNKT cell anergy and glycolipid toxicity, is the direct incorporation of glycolipids into bacterial membranes[87]. Further strategies for the improved delivery of glycolipid antigens could also be the use of specific-antibody/CD1d fusion proteins that are loaded *ex vivo* with α-GalCer. The administration of α-GalCer pulsed CD1d protein fused with an anti-HER2 single chain antibody Fv fragment (scFv) induced a potent anti-tumor response in a mouse tumor model using HER2-expressing B16 melanoma cells. Importantly, repeated injections of this fusion protein led to sustained iNKT cell activation with reduced anergy induction[88]. The group of Thapa et al. avoided α-GalCer-induced anergy with poly-lactic acid based nanoparticles conjugated with α-GalCer[89]. Recently, Gehrmann et al. reported that exosomes loaded with α-GalCer and ovalbumin activated iNKT cells, overcame anergy induction, and amplified tumor-specific adaptive immune[30]. Based on these interesting findings, our lab started to investigate if the use of dexosomes (exosomes originating from DCs) can be used as a delivery vehicle for α-GalCer to extend iNKT cell activation, reduce the subsequent anergy and increase anti-myeloma responses in the 5T33MM murine model.

Although this research is still in its infancy, the first *in vivo* results looks promising. More work is needed on optimizing the directed delivery of iNKT cell activators, which may allow the avoidance of anergy and result in the full therapeutic benefit without provoking unwanted toxic effects.

Finally, a critical number of general issues need to be considered in immune-targeted cancer therapy and are thus also concerning the iNKT cell-based immunotherapeutic strategy in MM. The first is the time point of administration. The optimal therapeutic window should be at the beginning of the disease when the cancer is starting to develop and not aggressive yet (as performed in this project in the 5T33MM murine model and in newly diagnosed MM patients), or when patients are in minimal residual disease after responding well to conventional treatments. At this point, patients are not immune compromised making them susceptible to immune stimulation. As seen in our 5T33MM murine model, iNKT cells are only responsive in a low tumor burden setting. Secondly, the expression of immune checkpoint receptors and the presence of their cognate ligands on tumor cells or other immune cells within the tumor microenvironment is also an important point to mention. A number of studies namely demonstrated a better efficacy with immune checkpoint blockade in patients who had high levels of CTLA-4 and PD-1 on immune cells in the tumor microenvironment and high expression of CTLA-4 and PD-L1 ligands on the tumor cells [90–93]. Furthermore, neo-antigens created by the multiple somatic mutations positively correlated with a response to PD-1 inhibitors in non-small cell lung cancer and CTLA-4 inhibitors in melanoma[94,95]. The main challenge here will be the development of diagnostic tools that will enable to stratify and select the appropriate patient population; and the designing of biomarker strategies that would give the potential to predict the response to therapy to maximize treatment efficacy. Finally, it is also evident that the successful deployment of immunotherapy such as the iNKT cell-based immunotherapeutic strategy will require a combined approach, in which specific targeting of MM cells is paired with tactics reversing the hyporesponsive anergic milieu. Meaningful synergistic effects and long-term survival benefits are anticipated with such strategies. However, precautions should be considered so that these combinations are well tolerated.

In conclusion, our data contribute to the understanding of iNKT cell biology in MM and has given us more insights in the concept of anergy impeding iNKT cell-based immunotherapy. Pursuing the above mentioned approaches, noting and addressing their limitations, will lead to the improvement of iNKT cell-based immunotherapy which will be beneficial for the therapy of MM patients.

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